STRUCTURAL DETERMINANTS OF STABILITY TO PROTEOLYSIS, PROCESSING AND IMPACT ON ALLERGENIC POTENTIAL OF NON-SPECIFIC LIPID TRANSFER PROTEINS

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ABSTRACT

Lipid transfer proteins (LTPs) are a class of low molecular weight hydrophobic conserved proteins comprising four intramolecular disulphide bonds making the structure very resistant to proteolysis and harsh food processing conditions. These proteins are identified as strong allergens sensitizing through the gut and share epitopes with LTPs from closely related species. Peach LTP, Pru p 3 is the primary sensitizer in the Mediterranean area being the most frequent food allergen. Wheat LTP, Tri a 14 is a relatively weak allergen with a very low prevalence. The study here compares the structural properties of these proteins and their resistance to various digestive and processing processes. Ligand binding experiments showed that Pru p 3 binds to ligands more strongly than Tri a 14. The gastroduodenal digestion of these LTPs revealed that both are stable to gastric digestion and while Pru p 3 is susceptible to duodenal digestion, Tri a 14 digestion is negligible. Ligand binding did not affect the digestibility of Pru p 3 but improved the duodenal digestibility of Tri a 14. The IgE binding studies using sera from peach allergic individuals confirmed that both Pru p 3 and its digestion fragments in the presence and absence of ligand were IgE reactive.

Model processing conditions were employed to treat these LTPs. It was found that heat treatment destroys the secondary structure of Pru p 3 at 121°C and slightly affects that of Tri a 14. Heat treatment also increased the susceptibility of Pru p 3 to gastric digestion while Tri a 14 was less affected. The IgE binding studies showed that heat treatment of Pru p 3 appeared to reduce its IgE recognition while its digestion fragments lost all of their IgE reactivity.

To investigate the effect of the food matrix on the digestibility of these LTPs, peach peel containing Pru p 3 and wheat flour containing Tri a 14 were digested under simulated conditions. It was found that they were resistant to proteolysis in their native matrices. Effect of heat treatment to the food matrix again confirmed that both of these proteins were more stable to heat in the matrix and were less digestible.

In conclusion, this study shows that there are factors in food matrices which enhance structural stability of LTPs to both processing and digestion. Thus factors such as the effect of food matrix and effect of processing should be taken into account in assessing the allergenic risk posed by foods and not simply rely on data from purified proteins.

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ABBREVIATIONS

1D: One dimensional
2D: Two dimensional
BCA: Bicinchoninic acid
BLG: Beta lactoglobulin
bp: Base pairs
BSA: Bovine serum albumin
CD: Circular Dichroism
CNBr: Cyano-bromide
CPA: Cis-parinaric acid
CV: Column volume
DTT: Dithiothreitol
E.coli: Eschericia coli
EDTA: Ethylene – di – amine tetra acetic acid
ELISA: Enzyme linked immunosorbent assay
GI tract: Gastrointestinal tract
HCl: Hydrochloric acid
HPLC: High performance liquid chromatography
IC: Inhibitory concentration
IFR: Institute of Food Research
IgA: Immunoglobulin A
IgD: Immunoglobulin D
IgE: Immunoglobulin E
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IL: Interleukin
IR: Infrared
JIC: John Innes Centre
LDS: Lithium dodecyl sulphate
LTP: Lipid transfer protein
MALDI – ToF – MS: Matrix assisted laser desorption ionization – Time of flight – Mass spectrometry
mdeg: Milidegree
MES: 2 (N-morpholino) ethanesulfonic acid
MHC: Major histocompatibility complex
MS: Mass spectrometry
NaOH: Sodium hydroxide
OAS: Oral allergy syndrome
OPD: Orthophenylene diamine dihydrochloride
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate buffer saline
PBST: Phosphate buffer saline + 0.1% (v:v) Tween 20
PC: Phosphatidyl choline
PCR: Polymerase chain reaction
Pru p 3: Prunus persica 3
PVPP: Polyvinyl – polypyrrolidone
Q-ToF: Quadrupole time of flight
RPM: Rotation per minute

RPM: Rotations per minute
SDF: Simulated duodenal fluid
SDS: Sodium dodecyl sulphate
SDS-Sodium dodecyl sulphate
SGF: Simulated gastric fluid
SSF: Simulated salivary fluid
TFA: Trifluoro acetic acid
Th: T helper cell
TMB: 3,3',5,5' – tetramethyl benzidine
Tri a 14: Triticum aestivum 14
UV: Ultra violet
v:v: Volume by volume
Vis: Visible
w:v: Weight by volume
w:w: Weight by weight
WDEIA: Wheat dependant exercise induced anaphylaxis
WHO: World health organization

SYMBOLS

°C: Degree celcius
µg: Microgram
µL: Microlitre
µM: Micromolar
cm: Centimetre
Cu: Copper
Da: Dalton
g: Gram
kDa: Kilo Daltons
kPa: Kilopascal
kV: Kilovolts
m/z: Mass to charge ratio
M: Molar
min: Minute
mM: Milimolar
mm: Millimetre
Mr: Molecular weight
N_2: Nitrogen
NaCl: Sodium chloride
nm: Nanometre
OH: Hydroxy
pH: Negative log of the hydrogen ion activity
pI: Isoelectric point
ppm:Part per million
V: Volts

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PUBLICATIONS and TRAINING

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2. **Mills E. N. C.**, Abdullah S. U., Alexeev Y., Mandalari G., Rigby N. M., Salt L. A., Sancho A. I., Watson A. D., Wickham M., Mackie A. R. (2009) The role of allergen structure, food matrix and processing in food allergy *(Oral presentation) The XXVIII Congress of the European Academy of Allergology and Clinical Immunology, Warsaw, Poland, 6-10 June 2009.*
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9. **Abdullah S. U., Alexeev Y., Johnson P. E., Rigby N. M., Watson A., Mackie A. R., Mills E. N. C.** (2011) Ligand binding increases susceptibility of non-specific lipid transfer protein to gastroduodenal proteolysis by increasing polypeptide mobility *(In preparation).*
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1 Introduction

The immune system protects higher organisms against the harmful substances in the environment, invaders such as microbial pathogens, and defective cells such as tumour cells. A healthy immune system is intelligent and can identify the difference between the "invader" and "friend". It achieves this through layers of systems. The first line of these is the physical barrier between an organism and the environment such as the stratum corneum of skin and mucus layer lining epithelial surfaces of the gastrointestinal, respiratory and urogenital tracts. The immune system is constantly surveying the environment across this barrier through adaptive immune responses which can generate specific response for environmental agents. In most cases, this removes or inactivates the harmful agents and remains functional afterwards providing a "memory" of the encounter. Such adaptive responses are complemented by innate immunity provided by, for example many kinds of antimicrobial peptides called defensins generated by the human body in response to attack by infectious organisms that restrict the activity and/or growth of micro-organisms (Yang et al., 1999). Other mechanisms include phagocytosis, the process of engulfing the solid particles by macrophages which enzymatically hydrolyze them, and complement, a system of small inactive zymogens circulating in blood that triggers the activation of proteases in the system and leads to release of cytokines (Aderem and Underhill, 1999).

In some cases, the immune system loses its "intelligence" to some extent and identifies substances, like foods or a component of them, as a harmful invader. In this way, the body initiates a series of responses to remove this component which is not necessary, which can initiate as adverse reaction to food of one type which has been termed as hypersensitivity reaction (Descotes and Choquet-Kastylevsky, 2001). During the 1920s, the term "atopy" was introduced (Coca and Cooke, 1923) to define an abnormal response associated with immediate type skin reactions like wheal and flare, which rapidly occur in individuals within minutes or hours after exposure to the trigger factor. Clinically this type of reaction was defined for hay fever, which is characterized by symptoms like sneezing, runny nose and itching following exposure to environmental factors like pollen or dust. It can also be accompanied by bronchial asthma, a condition involving constriction and inflammation of the lining of airways, as well as excessive mucus production. On further investigation, Coca and Grove (Coca and Grove, 1925) reported some heat labile components that they called "reagins" which were responsible for atopy, but they were unsure whether reagins were produced as result of immunological stimulation. In the following decades, the conditions such as asthma, hay fever, atopic dermatitis (inflammation of skin), perennial rhinitis (irritation and inflammation of the nasal

passage) and food allergy became closely associated, and it was found that levels of antigen specific immunoglobulin E (IgE) were increased in these conditions (Johansson et al., 2001).

Subsequently, Coombs and Gell (1968) proposed a classification of adverse reactions into four types, known as type I, type II, type III and type IV, based on the exposure to drugs but which could also be used for other conditions including allergies.

Type I reactions are acute (i.e immediate), occurring with minutes of exposure to a trigger. They share common clinical features, manifested mainly as urticaria and angioedema, along with cardio-respiratory shock (anaphylaxis), asthma, rhinitis or conjunctivitis. Individuals previously sensitized have a state in which mast cells and basophils are rich in IgE bound to high affinity receptor located on the cell surface. On exposure to a multivalent antigen, these IgE molecules are cross linked by antigen which triggers degranulation of the mast cells and basophils and release of the mediators. The symptoms result from action of mediators like vaso-active amines, such as histamine, released from mast cells and basophils. This is followed by synthesis of secondary mediators like thromboxanes, prostaglandins and leukotrienes.

In contrast, type II reactions are delayed in nature and involve cytotoxic antibody-mediated (IgM, IgG) reactions and can proceed via two different mechanisms. Firstly, macrophages, neutrophils and eosinophils can directly attack an antigen by homing in on antigen-bound IgM and IgG. Another mechanism involves activation of the complement classical pathway, where hydrolases cause cell lysis. Commonly, such reactions include haemolytic anaemia, granulocytopenia and thrombocytopenia and may occur in hours or even days after exposure to a trigger.

Type III reactions involve tissue injury caused by the soluble immune complexes of antigens formed with specific IgG and in some cases, insoluble immune complexes formed with specific IgM. When exposed to an abundant mass of antigen, a soluble antigen-IgG complex is formed. In normal functioning, these complexes are removed by liver. However, if they are not destroyed in the liver, they are carried by the circulation into body tissues such as lung, joints, kidneys and skin, where they become deposited and cause inflammatory reactions. The resulting lesions contain neutrophils, the immune complex and some complement compounds like C3a, C4a and C5a.

The type IV reaction is another delayed reaction but involves cellular, rather than antibody reactions. Contact dermatitis and urticaria are good examples of this type of adverse reaction. Environmental factors like drugs, cosmetics and other chemicals are involved. Usually, symptoms may appear within 4 to 8 hours of exposure but may take up to 2 to 14 days to appear.

Whilst the Gell and Coombs classification has been important in helping to define different types of allergy, it has some limitations. For example allergic reactions to drugs do not fit in this classification, one reaction may involve more than one mechanism, and one drug may induce several hypersensitivity reactions via several mechanism (Metcalfe et al., 2008).

In 1968, WHO announced the fifth immunoglobulin IgE, which was proposed in the same decade to be responsible for the classic reaginic activity first described by Coca and Cooke (Coca and Cooke, 1923). Later on, the term "atopic allergy" was introduced by Pepys (1975) referring the inhalant allergens only. The term "atopic" now is being used to define the hypersensitivity reactions that are IgE mediated.

In more recent times (Johansson et al., 2001), the term "hypersensitivity" has been defined as the reaction(s) initiated by exposure to a defined stimulus at a dose tolerant to a healthy person, in an individual whose symptoms are reproducible. It encompasses both immunological and non-immunological reactions but excludes infections, autoimmune and toxic reactions. The definition also excludes drug and multiple chemical sensitivities, reactions to amalgam, electromagnetic waves and other physical factors. Allergic hypersensitivity is the reaction that essentially involves an immunological mechanism (including cell mediated reactions such as celiac disease and IgE mediated reactions) while non-allergic hypersensitivity has no proven immunological mechanism and includes conditions such as lactose intolerance, a condition caused by a lack of the enzyme lactase required to digest this sugar (Johansson et al., 2001).

According to the classification of food allergy, it is divided into two types depending upon the mechanism involved in the clinical expression. The first type so called "type 1 food allergy" is the adverse food reaction mediated by IgE. This is an immediate type reaction commonly expressed as gastrointestinal hypersensitivity, oral allergy syndrome, acute urticaria, angioedema, allergic rhinitis, asthma and anaphylaxis. The second type of allergy is a non-IgE mediated allergy mediated by T-cells, eosinophils and IgG. Major clinical expressions are coeliac disease, dermatitis herpetiformis and dietary protein-induced enterocolitis (Metcalfe et al., 2008).

1.1 Food allergy

Food allergy is major health problem in western countries with increasing prevalence with time (Sicherer et al., 2003). Many food allergens are glycoproteins in nature ranging 9-70 kDa molecular weight with high abundance in foods and possessing multiple epitopes for IgE (Bannon, 2004). The clinical symptoms developed depend upon various factors like the characteristics of the allergen,

and the level of exposure. Similarly the food matrix and whether an allergen was ingested alone or in combination with other foods may all affect the absorption of allergen which in turn plays an important role in developing reaction. Other factors including alcoholic beverages, aspirin or exercise also affect clinical presentation (Asero et al., 2007).

Historically, eight foods were described as being responsible for more than 90% of allergic disorders all over the world. These foods are soy, egg, milk, wheat, peanut, tree nut, fish and shellfish, although other food groups like fruits and vegetables have also been recognized to cause allergy (Bush and Hefle, 1996). Atopic diseases especially respiratory allergies are being raised in many developed countries including UK and USA. Food allergy is more prevalent in children than adults affecting 6-8% in UK and USA. A general pattern of food allergy around the globe is given in Fig. 1.1. In general, the prevalence of food allergy among countries like US, Canada, UK, Australia, New Zealand, rest of Europe including Scandinavian and Mediterranean countries, Japan, Korea and China has been reported from as low as 1-2% to as high as 10% of the population studied. The self-reported food allergy was 12% in children while 13% in adults. On the basis of testing including double blind placebo controlled food challenge (DBPCFC) and positive history, the prevalence was found to be 3% (Sicherer, 2011). Among various foods, prevalence of allergy to peanut (0.75%), milk (0.6-3.5%), egg (0.3-1%), fish (0.2-0.6%) and shellfish (0.6-1.1%) is reported (Sicherer, 2011, Rona et al., 2007).

Fig. 1.1: Patterns of food allergy in developed countries of different continents of world. Adopted from (Poel et al., 2009).

Cow's milk allergy is predominantly found in infants and young children although around 85% of them develop tolerance by the age of five (Shek et al., 2004). The main milk allergen is the native milk protein casein. Many cows' milk allergic children can tolerate hydrolyzed milk but for some only, amino acid formula is hypo allergenic. The symptoms are the same for all foods as a rule. Allergy to egg is also found frequently in children but like milk allergy, they can usually tolerate it by 3-5 years of age. Soybean allergy is found in early childhood and is due to the soy proteins as individuals usually tolerate soya oil and lecithin-containing foods. Allergy to peanuts is a most common allergy found in western populations, especially North America whilst other nut allergies, such as hazelnut, are found in Europe. Allergy to fish and shellfish is observed especially in Asia (Rosenlund et al., 2011). Since the subject of this thesis is comparison of allergens from fruit (peach) and cereals (wheat), these will be described in more detail.

1.1.1 Fruit allergy

The main reason for fruit being allergenic is the presence of immunologically active proteins either homologous to pollen allergens, which result in cross-reactivity such as in Central and Northern Europe, or resistance to proteolysis sensitizing through the gastrointestinal tract. The labile allergens such as those cross-reactive to Bet v 1 and Bet v 2 sensitize through oral mucosa or through respiratory tract. Allergy to fruit may be due to primary sensitization to the food itself or as a result of prior sensitization to pollen or latex. The major allergic reactions to fruits have been reported as a condition known as oral allergy syndrome (OAS), sometimes associated with asthma, wheezing and rhinitis, eczema being a less widely reported symptom (Rosenlund et al., 2011). Major allergenic fruit include Rosaceae species, such as apple, pear, stone fruits such as peach and plum, together with kiwi (Fernández-Rivas et al., 2008). The pattern of fruit allergy varies within Europe shows regional variation. For example, in Northern and Central Europe, fruit allergy is largely associated with birch, alder and hazel pollen allergy, and gives rise to mild symptoms and has been termed class II allergy (Eriksson et al., 1982, Ebner et al., 1995, Ortolani et al., 2000). Several Rosaceae fruits such as apple are examples of this cross-reactivity as they failed to inhibit birch extract with a serum pool of patients allergic to apple. This exlains that birch allergens were primary sensitizer while elicitation occurred with apple allergen (Andersen et al., 2009). In Western Mediterranean areas such as Spain, sensitization to birch pollen is absent due to a lack of birch trees and fruit allergy is more severe. It appears to result primarily from sensitization to peach itself, and hence termed class I allergy. However, an association with pollen from mugwort and plane trees has also been noted (Andersen et al., 2009).

1.1.2 Cereal allergy

The top most consumed cereals in the world are wheat, rice and maize with wheat being the most important. Two major types of IgE-mediated allergies to wheat have been described, one known as bakers' asthma, the other wheat-dependant exercise induced anaphylaxis (WDEIA). Bakers' asthma is an occupational allergy associated with inhalation of wheat flour, especially in wheat milling factories and bakeries where flour is abundant in the air as dust. The prevalence of wheat allergy in Europe and United States is reported as low as 0.2-0.9% in adults while 0.4-1.3% in children as determined by questionnaires filled by the population under study while another study reported it as high as 0.4-4% in adults as determined by non-specific IgE binding to wheat (Morita et al., 2012).

Maize allergy is not a very common allergy and is due to the presence of LTP which is cross reactive to peach LTP (Venter et al., 2008). Rice allergy is common in Eastern Asia (Suvarna, 2008) and is due to the presence of albumins ranging 14-16 kDa belonging to the family of α-amylase and trypsin inhibitors (Tsuji et al., 2001).

1.1.3 Immunological basis of food allergy

The allergic response is considered to be a result of priming of the immune system resulting in sensitization where in most cases of healthy individuals the response is suppressed by the mechanism of oral tolerance. This allergic response occurs when mature lymphocytes in lymphoid tissues either lose their function or become hyporesponsive. Tolerance is achieved by administration of allergen through oral route where this allergen gains access rapidly to mucosal and systemic lymphoid tissues. Intestinal epithelial cells also play a very important role in induction of tolerance. In case of dendritic cells, the tolerance is achieved at a stage where these cells present soluble antigen but minimal CD80 and CD86 which are required for allergic response (Sampson, 2003, Chehade and Mayer, 2005). Individuals who loose their tolerance to certain foods become allergic, a process which can be divided into two phases; the sensitization phase and the elicitation phase.

1.1.3.1 Factors affecting antigenic response

According to Strobel (Strobel and Mowat, 1998), the antigenic response is dependant upon various factors affecting the direction of antigen processing either towards an allergic reaction or induction of tolerance.

Nature of antigen: The nature of the antigen plays a very important role in development of antigenic responses. It was observed (Sun et al., 1994) that thymus dependant soluble antigens, when administered through oral route follow the tolerance mechanism rather than antigenic mechanism. Similarly, particulate or replicating antigens such as *E. coli* often induce active immunity and are less tolerant because of their preferential uptake by M-cells and subsequent antigen processing (Pierre et al., 1992).

Dose of antigen: It was observed that the frequency and size of dose plays a very important role in generating tolerance with varying effects on the individual immune components. Single administration of antigen at higher doses suppresses tolerance and induces allergic responses while multiple administrations of small doses generally induce tolerance (Garside et al., 1995). Continuous administration through physiological routes like drinking water leads to profound induction of tolerance. The absorption of an intact form of an allergen or the fragments containing a B- or T-cell epitope is a key factor affecting the allergy/ tolerance of the food (Melamed et al., 1996).

Genetic Background: Studies using mouse models clearly demonstrate that antigenic responses are also dependant on the genetic background of the individual as some strains tolerate a number of antigens while some not. The clearance of antigen from blood circulation is also influenced by genetic structure of individual and hence, affects the degree of tolerance (Strobel and Mowat, 1998, Stokes et al., 1983).

Host Maturity: The age of the susceptible individual is a common factor observed in allergic population. For example, it is observed that prevalence of allergy to some foods like milk, and egg is greater in children than adults.

1.1.3.2 Sensitization

The first phase of allergic reaction is called sensitization in which the host produces an immune response to an invader or environmental agent such as allergen. The allergen is engulfed by antigen presenting cells like dendritic cells and macrophages found after crossing the epithelial barrier. There are various routes through which antigen can enter into the body. This may be through inhalation, skin contact or ingestion. The dendritic cells found in the epithelial surfaces (respiratory, gastrointestinal tract or skin) engulf these allergenic proteins and process them into smaller peptides. These peptides are then presented to T-cells which is polarized to interleukin producing

Th2-cells. These interleukins are then recognized by B-cells which then produce IgE circulated through blood and surfaced bound to mast cells (Fig. 1.2).

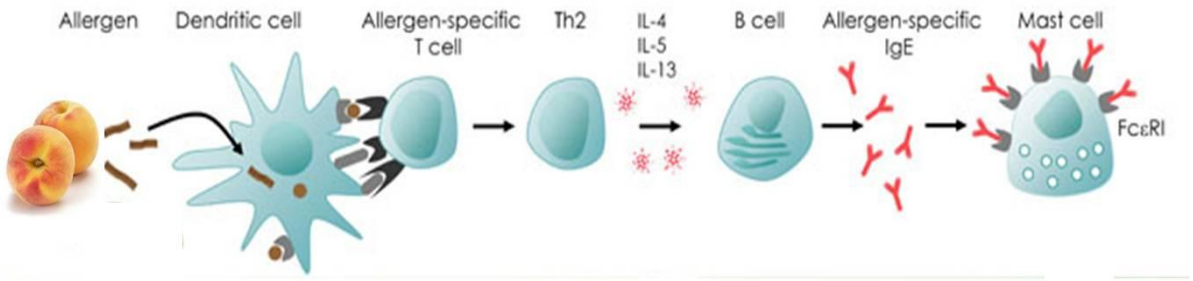


Fig. 1.2: Sensitization to allergens such as those from peach result in production of mast cells with allergen specific IgE bound on the cell surface.

Another way of encountering these allergens to immune system is in the gut through the M-cells, located in specialized structures known as Peyer's patches that deliver antigen to underlying dendritic cells. These antigens may also encounter T-cells, or macrophages in the lamina propria or may go to the circulation and hence be available to interact with the immune system (Chehade and Mayer, 2005). The major events in this allergic sensitization phase are antigen presentation, T-cell polarization and B-cell maturation to produce allergen specific IgE.

1.1.3.2.1 *Antigen presentation*

A heterologous group of cells present at peripheral tissues that are in contact with the environment, like the gut mucosa and skin dendritic cells, comprise about 1-2% of the total population of the cells in tissue. They are produced in an immature and inactive state in bone marrow. Once they are exposed to an antigen, they mature and are deposited to the peripheral tissues. During maturation, they adopt a shape of branched projections, and hence are named as dendritic cells (dendrite being the latin for branch) (Banchereau and Steinman, 1998). They can take up and process antigen and play a key role in activating and polarising T-Cell response.

The process by which antigens are taken up and processed by a group of cells including dendritic cells, macrophages and B-Cells, is termed antigen presentation. Dendritic cells are known to capture pathogens, infected cells, dead cells or their products or any other foreign matter to undergo antigen presentation. Initially, these cells engulf the materials to form an intracellular vacuole after which they are fused with endoplasmic reticulum and processed in the endosomal compartment. Endosomal proteases hydrolyze these proteins and fragment them into smaller peptides which are

then become bound to the MHC class II (majorhistocompatibility complex class II) molecules. These then present them on the cell surface from where they are recognized by T-lymphocytes via T-cell receptors located on CD4+ helper T –cells which are activated as a consequence of this complexation. A graphic representation of antigen presentation is given in Fig. 1.3.

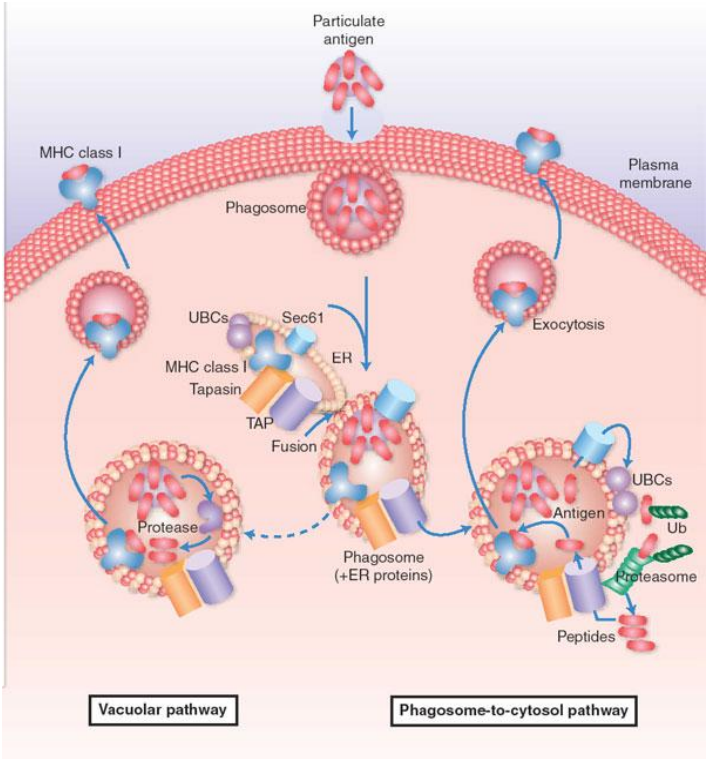


Fig. 1.3: Antigen presentation by a dendritic cell. The phagosome is fused with endoplasmic reticulum containing which then process antigen with its antigen presenting machinery and express them at cell surface through MHC (Rock, 2003).

1.1.3.2.2 *T-cell polarization*

A type of white blood cell, T-cells are an essential component of the immune system and contributes to cell-mediated immunity. They can be classified into different types. Thus, helper T-cells secrete cytokines and possess CD4 receptors on their surface, cytotoxic T-cells that destroy for example infected cells and possess CD8 receptors at their surface, whilst memory T-cells persist after infections have cleared and proliferate rapidly following repeated exposure of the original stimulating antigen. Memory T-cells possess either CD4 or CD8 receptors. Other classes include regulatory and suppressor T-cells that manage immune responses and are characterized by CD4 and CD25 receptors, whilst natural killer cells recognise glycolipids.

The activation of T-cells depends upon their binding via the T-cell receptor to a short antigen-derived peptide presented by the MHC class II molecules on a partner cell like a dendritic cell or a

B-Cell. The CD8 receptor binds shorter peptides 8-9 amino acids in length while CD4 recognise longer peptides. The stimulation of T-cell activation occurs by CD28 on its surface while its co-stimulation is based upon recognition of CD80 and CD86 present on the dendritic cell. They produce specific cytokines and in allergic individuals the expression pattern is skewed to a Th-2 phenotype mostly interleukin-4 (IL-4), IL-5 and IL-13. In addition to antigen specific signals presented by MHC class II, the T-cell polarization is thought to be driven by CD80 and CD86 on dendritic cells, triggering the expression of CD28 on T-cell. These two signals activate T-cells to rapidly express CD40L that ligates with CD40 on dendritic cell. At this stage T-cells produce high level of cytokines, which polarizes it through third signal called polarizing signal into either Th1 or Th2 cell (Kapsenberg, 2003). This third signal is dendritic cell dependant i.e. how it was activated. The Th2 cells produce IL4, IL5, IL9, IL13 along with the expressed CD 40 L which play important part in stimulation of B-cell and production of high level of IgE (Guermonprez et al., 2002). In the case of allergens, T-cells become polarized into Th-2 cells in response to IL-4 which redirects native Th cells to Th-2 phenotypes, while IL-10 suppresses the polarization in Th-1 direction (Callard et al., 1996). A schematic representation of T-cell polarization is given in Fig. 1.4.

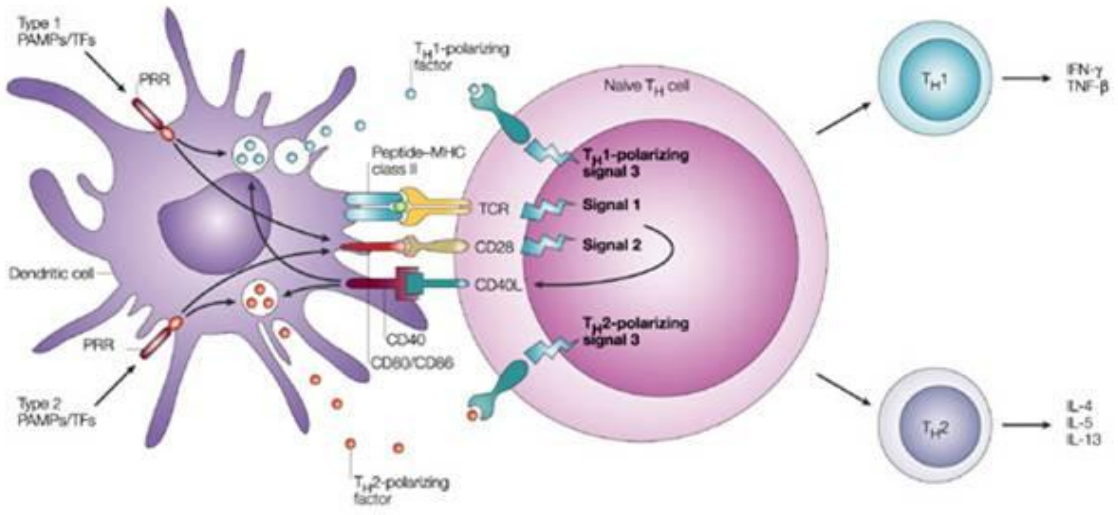


Fig.1.4: T- cell polarization either into Th1 or Th2 cells. PRR: pattern recognition receptors; PAMP: pathogen associated molecular pattern; TF: inflammatory tissue factor (Kapsenberg, 2003).

1.1.3.2.3 *B-cell maturation and immunoglobulin production*

Antibodies (also known as immunoglobulins, Ig) are high molecular weight (~150 KDa) "Y" shaped defensive globular glycoprotein molecules. They are produced by a healthy immune system specifically by B-cells and are present in serum and other tissue fluids of mammals, like lymph.

They have a conserved basic structure, with two large heavy chains and two light chains joined by disulphide bonds. They are found in monomer, dimer or pentamer forms. Although, the basic structure of these antibodies is similar, the tip of these antibodies, where the antibody binding site is located, is found in billions of slightly different forms, allowing them to recognise billions of different antigens found in nature. This variation is conferred by the hyper-variable region of the antibody. The unique part of the antigen that is recognized by an antibody is known as an epitope.

There are five different classes or forms of antibodies produced by mammals' immune system which are commonly known as immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), immunoglobulin G (IgG) and immunoglobulin M (IgM). IgG, IgE and IgD are found in monomeric forms while IgM is found in a pentameric form. The IgA is found in monomeric, dimeric and pentameric forms (Roitt et al., 1998).There are two forms of antibodies, a soluble form that is found in the blood and other body fluids, while a membrane bound form attached to cell surface of B-cells. They are produced in response of attack by foreign intruders such as microorganisms, bacteria, virus, parasites and other foreign materials. After their synthesis, they circulate in the blood and either become bound by other immune cells, like tissue mast cells and blood basophils, or remain free in the lymph.

IgG is the most abundant immunoglobulin, accounting 70-75% of the total immunoglobulin in serum and is regarded as antitoxin. It is equally distributed between intravascular and extravascular pools. IgA is the second most abundant antibody accounting 15-20% of the total immunoglobulin synthesis. It is found in monomeric form in humans while in mostly dimeric forms in other mammals. Mostly it is found in seromucous secretions like saliva, milk and tracheobronchial secretions as well as the gut. IgM is another abundant immunoglobulin that accounts 10% of total immunoglobulins. In general, it is found in an intravascular pool and its production is associated with microbial infections. IgD is found in very low levels, usually <1% and is largely present on the surface of B-cells. IgE is found on the cell surface of mast cells and basophils and is produced in response to parasitic infections, or to environmental agents in allergic disease (Roitt et al., 1998).

Immature B-cells are produced in bone marrow in case of mammals or in bursa of fabricius in birds and are key components of the immune system since they are responsible for synthesising antibodies. Initially, when these cells are formed, they migrate via the circulatory system to certain areas of the body like the spleen and lymph nodes. There they differentiate in one of two ways; the first one is antigen independent where the genes for antibodies are selected for rearrangement and expression which leads to production of memory B-cells. The second involves antigen dependent

activation, proliferation and differentiation to form antibody producing plasma cells. Differentiation occurs as a consequence of signals from antigen, dendritic and T-cells (Tanabe, 2008). In case of the antigen being an allergen, the B-cells respond to the stimulation produced by these cell types to produce allergen specific IgE which circulates in blood and received by major recipients in allergic reactions like mast cells and blood basophils through Fc receptor. Both the IL4 and IL13 secreted by Th2-type T-cells induce B-cells to proliferate, activate and express CD23 along with switching from antibody production from IgM to IgE. The whole process is called as "sensitization" as the individual doesn't show any allergic symptom at this stage (Tanabe, 2008). A schematic representation of this process is given in Fig. 1.5.

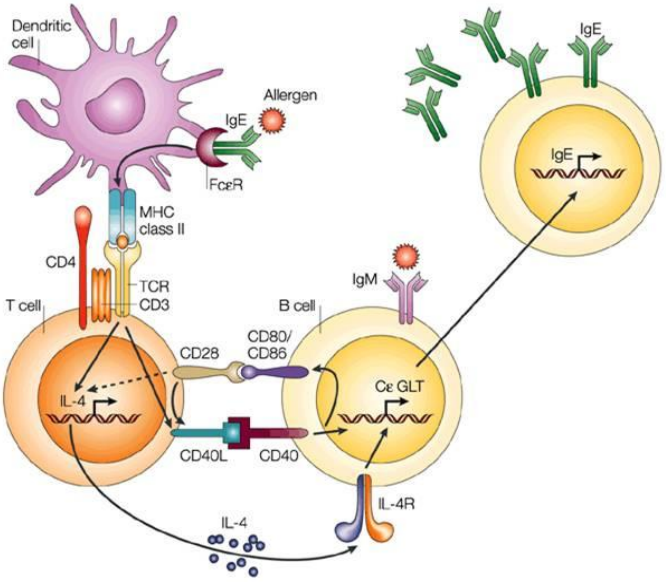


Fig. 1.5: Antibody production by B-cells as a function of T-cell response dependant on antigen presentation (Ahrazem et al., 2007).

1.1.3.3 Elicitation

The second phase of the immune response in food allergy is responsible for the expression of allergic disease in allergic reactions. The main event in this phase is degranulation of mast cells and basophils which triggers physiological responses such as vasodilation. The step is dependant upon the multivalency of the allergen to which the sensitized individual is exposed. When the multivalent allergen enters the blood it is recognized by IgE bound to the surface of the cells such as mast cells and cross link them. This aggregation of IgE is responsible for the release of mediators from mast cells and basophils where they reside in their "pro" form, and includes histamine, cytokines,

interleukins, serotonins, leukotrienes and prostaglandins. The release of these mediators into the tissues results in the symptoms of an allergic reaction including inflammation, vasodilation, excess mucus secretion, smooth muscle contraction, itching amongst others (Tanabe, 2008). A schematic representation of elicitation is given in Fig. 1.6.

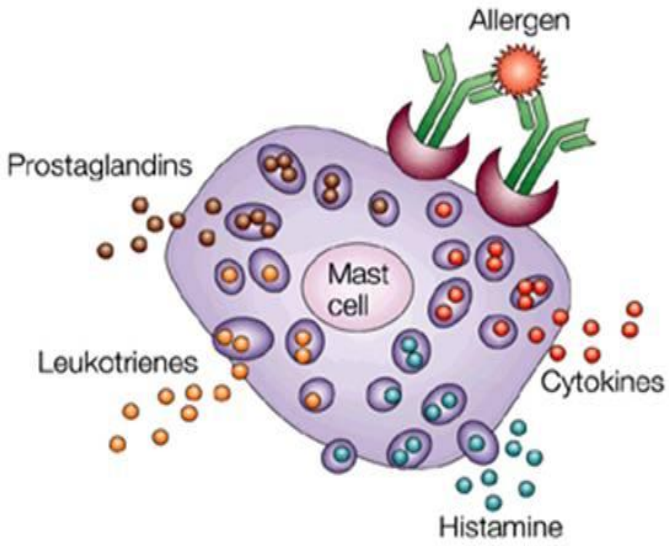


Fig. 1.6: Degranulation as a result of elicitation of allergic response upon re-exposure of allergen (Geha et al., 2003).

1.1.4 Diagnosis of food allergy

Initially, a history of the disorder is taken including which food caused the reaction. The length of time between ingestion and symptom development, previous observations of reactions after eating the same food, all need to be noted. Other factors that are important are whether the symptoms are associated with exercise (Sampson, 2003). In general, it is difficult to correctly identify a problem food as the patient may be eating a mixture of food while the symptoms may arise sometimes after eating. Therefore, it is important to identify whether the symptoms are IgE dependant or non-IgE mediated (Sicherer, 2002). The diagnosis of food allergy is based upon two classes of diagnostic methods, *in vitro* tests commonly performed to identify IgE in serum of patients and *in vivo* tests.

1.1.4.1 Serological analysis *in vitro*

In vivo tests are expensive and time consuming to perform. Therefore, *in vitro* laboratory tests offer some advantages. There are several laboratory tests that used to determine food specific IgE in

serum, including radio allegro-sorbent test (RAST), enzyme linked immunosorbent assay (ELISA), immunoblotting and histamine release assay.

1.1.4.1.1 *Radioallergosorbent/ Enzyme allergosorbent test (RAST/ EAST)*

Initially developed in 1970 and commercialized in 1974, RAST was, for a long time the method of choice for determining specific IgE. The test is simple to perform and needs a disc, usually made of microcrystalline cellulose that is coated and covalently crosslinked with an extract of the food to be tested. The disc is incubated for a certain period of time with the serum of the patient, bound IgE being detected with radioactive iodine (I^{125}) labelled antihuman IgE. The method has been modified because of safety considerations associated with using radio-labelled reagents. Several equivalent methods have now been developed using fluorescent labelled antibodies, enzyme coupled monoclonal antibodies (EAST) and biotin coupled allergens (detected by fluorescent streptavidin). These have been accompanied by physical improvements like the use of solid-phase support systems to increase the binding capacity of allergens and automation (Sánchez-Monge and Salcedo, 2005). Sometimes, the serum is incubated with a varying amount of extract or purified allergen and hence inhibits fully or partly IgE-binding to the solid support to give inhibition tests (RAST/ EAST inhibition). However, the sensitivity of these tests is much less than RAST (Mills et al., 2006).

1.1.4.1.2 *Phadia CAP system*

An adaptation of the RAST principal is the commercial Phadia CAP system used to determine the total and specific IgE in the undiluted sera of patients. Solid bound allergens are allowed to react with the antibodies in the sample followed by detection of antibodies by labelled anti-IgE. The system is built around a new type of solid-phase, consisting of a flexible hydrophilic carrier encased in a capsule, ImmunoCAP. The carrier is a highly efficient disc that is made up of CNBr-cellulose that can adsorb at least three times more protein than a normal paper disc and around 150 times more than a tube coated with the same material providing a highly favourable condition for allergen antibody reaction with improved diffusion capacity. The system utilizes monoclonal and polyclonal anti-IgE antibodies labelled with β-galactosidase that generates fluorescence generally regarded as fluorescence immunoassay (FEIA). The system works in very low and very high concentration of allergen making its capability very improved. The clinical investigations proved its diagnostic value and confirmed the correlation between the serum concentration of IgE antibodies and allergic

disease (Bousquet et al., 1990) although it only determines the extent of sensitization and does not necessarily indicate if an individual has clinical allergy.

1.1.4.1.3 *Histamine release assay*

The histamine release test determines the IgE bound to basophils of a patient susceptible to allergy which is capable of triggering degranulation. It gives an *in vitro* measure related to *in vivo* reactions like skin prick tests (Sec. 1.4.2.1). The heparinised blood from an allergic patient is exposed to the susceptible allergen that initiates the cross linking of basophil surface bound IgE leading to degranulation of the cell. The assay follows this by measuring the amount of histamine release. Recently, a glass fibre is being used that absorbs histamine released with high affinity and selectivity. The histamine released was adsorbed with glass fibres and quantified by treatment with o-phthalaldehyde. The condensation reaction product is coloured and highly fluorescent and can be measured at 355/ 450 nm (Uchio et al., 2001).

1.1.4.2 *In vivo* tests

Whilst *in vitro* tests are simple to conduct, easy to control and mostly economical, they some times provide false positive results. Therefore, *in vivo* tests still have a place of importance and the final conclusion over whether a patient has a food allergy depends upon the results from such tests.

1.1.4.2.1 *Skin tests*

Skin tests are still the most widely used, simple and easy to perform tests that provide fast and, to some extent, quantified results. Initially it was regarded as a qualitative test but can be performed in a semi-quantitative fashion to give a measure of extent of reaction. The test is simple and needs no instrumentation which is perhaps, a strong reason to choose it for routine testing. A very small volume of glycerinated extract (~ 30 µ L) is dropped on to the skin, e.g forearms, of the patient and then pricked into the skin by a lance. The positive (histamine) and negative (saline) controls are also applied but at a distance apart to avoid the overlapping reactions. The appearance of a wheal of area at least 75% of the positive control (histamine) is regarded as a positive test (Pauli et al., 1996) while the minimum area should be 7 mm^2. Several factors can affect the results, such as the quality of the food extract, its freshness, prick-to-prick technique and use of other medications. Since fresh fruits and vegetables are rich in proteases, they may alter the allergen structure making extracts ineffective compared with fresh fruits. Thus an allergic food may show a negative test while a false

positive test may be due to clinically insignificant sensitization or cross-reactivity. Whilst skin tests are used as a primary tool to identify allergy, their results need coupling with some other tests to ensure the reliability of the diagnosis (Asero et al., 2007).

1.1.4.2.2 *Oral challenge and double blind placebo controlled food challenge tests*

Oral challenge tests are considered as the gold standard for confirming food allergy in a patient. The suspected food is given to the patient to eat with continuous monitoring of symptoms and measures such as blood pressure, pulse, respiration rate. Physical symptoms assessed include wheal, flare and the subjective symptoms experienced by the patient like itching, tightness in throat. The duration and the quantity of food required to produce such symptoms is also recorded (Ozcelik and Haytac, 2006). Again, food aversion is the problem associated with this test, an individual can react to placebo. This is minimized by introducing the double blind placebo controlled food challenge (DBPCFC). In this method, at least two meal samples are prepared, an active sample with potential allergen while the other is a placebo with the same colour, texture, appearance, taste, aroma and other physical parameters as the active dose. The homogeneity should be monitored by triangle tests where the difference is measured by identifying one from other in a taste panel where both the foods are tested at the same time (Bindslev-Jensen et al., 2004) although this is not always the case. The meals are administered to the patients in such a way that neither patient nor the physician knows which is placebo and which is active. Both the meals are administered on different days while the doses are started from a very small volume to the average daily serving with a time difference of mostly 15 minutes or until the symptoms start (Ballmer-Weber et al., 2007). The drawback of the test may be the masking of allergen in the complex environment of the food or other irrelevant changes that may happen between the time of preparation to the time of administration. Therefore, it is preferable to perform the challenge for some foods with fresh food (Asero et al., 2007). Again, the method lacks standardization and can be risky for patient (Bindslev-Jensen, 2001).

1.1.5 Allergen management

Previously, it has been suggested that exposure to allergenic foods in the early stages of childhood caused sensitization to allergens. It was thus hypothesized that avoidance of food allergens in the early stage of childhood by using hypoallergenic infant formulas may reduce the risk of food allergy at later stages of life by helping individuals to develop tolerance (Allen et al., 2009). However, the concept failed in terms of increased allergies including anaphylaxis to some foods such as peanuts as observed in Western countries due to a lack of tolerance.

In immunotherapy to treat allergies, traditional methods include injecting the allergen into the dermis to improve cellular and humoral mechanisms responsible for tolerance. The drawback of the technique is the direct exposure of allergen to the immune system which may elicit severe allergic reaction. Recent advances in this therapy involve introduction of engineered allergens into the dermis or oral administration of allergen either sublingual in the form of extract or ingestion along with a food vehicle to avoid this problem. All these methods start with very low doses and increase in quantity over time (Scurlock et al., 2009). Other methods of immunotherapy involve administration of heat denatured allergens, suppress Th2 mediated immunity in a general fashion for all allergens and the use of Chinese herbal medicine (Skripak and Sampson, 2008).

Since there is no cure at present, if someone has allergy, that person has to avoid their problem food. In common practice, food industries with a Good Manufacturing Practices (GMP) approach use classic hazard analysis critical control points (HACCP) to assure the removal of allergenic components not listed in the ingredients and label clearly the allergens that are the part of the formula. Parallel to this manufacturing process, there are various methods developed to identify the presence of allergens in the samples from various steps of processing to finished product (Ward et al., 2010).

1.1.6 Classification of food allergens

Allergens were originally classified by source as reported by International Union of Immunological Societies Allergen Nomenclature Subcommittee (Chapman et al., 2007). However, other criteria that have been used are the biological function of allergen, the protein fold/ family to which they belong. Among this class of proteins, those which were glycosylated showed higher activity against non-glycosylated forms. In cereals such as barley and rice, same amylase inhibitors along with trypsin inhibitors showed IgE reactivity. Other protein identified as allergens are wheat germ agglutinin and gliadins (Tatham and Shewry, 2008).

Wheat dependant exercise induced anaphylaxis (WDEIA) is associated with α, β, γ and ω-gliadins along with some other low molecular weight subunits of gluten proteins. This type of allergy is induced by ingestion of wheat and subsequent physical exercise and is not seen in the absence of any of the two factors (Tatham and Shewry, 2008). The major allergen causing this adverse reaction has been identified as ω_5 gliadin (Morita et al., 2003).

This introduction will use the latter, where allergens can be grouped on the basis of sequence identity and three dimensional structure into a protein family. For plant food allergens, these include

the major families namely the cupin, prolamin and Bet v 1 families together with minor families such as the profilins.

1.1.6.1 Cupin superfamily

This family of proteins is grouped on the basis of possessing a common 3D structure comprising a β-barrel. The barrel is composed of two conserved motifs each containing two β-sheets separated by two less conserved β-sheets and a small loop. Whilst the primary sequence of this family of proteins spans microbes and plants and shows weak homologies, the 3D structure is conserved. They have diverse functions including enzymes such as dioxygenase, germins and germin like proteins (GLPs), phosphomannose isomerise, oxalate decarboxylase, auxin binding proteins and nuclear proteins. The most widely found cupins are in the form of bicupins which include the seed storage globulins of the 11S – legumin type and 7S – vicilin type which were originally identified on the basis of their sedimentation coefficients. Other multicupins have also been reported in literature (Dunwell et al., 2004).

1.1.6.1.1 *7S Seed Storage Globulins*

These are high molecular weight disc shaped proteins found in trimeric form with the subunits ranging from 40 to 80 kDa. They lack cysteine residues and hence are not stabilized by disulphide bridges. Various members of this class of proteins undergo post – translational processing including proteolysis (such as pea 7S) and glycosylation (such as bean 7S, phaseolin) on the individual subunits. Many food allergens belong to this group including the major peanut allergen Ara h 1, and Jug r 2 from walnut (Hauser et al., 2008, Breiteneder and Radauer, 2004).

1.1.6.1.2 *Legumin Type 11S Globulins*

These are composed of a 20 kDa basic polypeptide which is disulphide linked to an acidic 35-40 kDa polypeptide. Each of these subunits is synthesized as a precursor and proteolytically cleaved in the seed prior to assembling into a hexameric structure comprising two trimers. Examples of allergens are Ara h 3, Ara h 4 from peanut and Cor a 9 from hazelnut (Breiteneder and Radauer, 2004, Hauser et al., 2008).

1.1.6.2 Prolamin superfamily

The family groups the proteins on the basis of an eight cysteine motif (8CM) proteins. They are characterized by high proline and glutamine content. All the proteins in this family are low molecular weight proteins with a conserved pattern of disulphide bridges. They share a similar 3D structure rich in α-helices. They are stable to heat and gastrointestinal digestion. Therefore, they may elicit severe anaphylactic reactions (Breiteneder and Radauer, 2004, Hauser et al., 2008). The family is subdivided into following groups.

1.1.6.2.1 *2S Albumins*

These are major seed storage proteins in dicotyledonous plants rich in arginine, glutamine, and asparagine along with eight cysteines in conserved pattern. Mostly, they are heterodimeric comprising subunits of 4 and 9 kDa joint by four conserved intermolecular disulphide bridges. They are characterized and identified by their sedimentation coefficient as 2S. They are mobilized during germination and hence act as nitrogen and sulphur donor as well as antifungal protein. They have high frequency of systemic allergic reactions. Allergenic forms include Ber e 1 from Brazil nut and Jug r 1 from English walnut (Breiteneder and Radauer, 2004, Hauser et al., 2008).

1.1.6.2.2 *Non specific lipid transfer proteins*

Monomeric proteins, the LTPs can be subdivided in to two classes, LTP 1 with molecular weight 9 kDa and LTP 2 with molecular weight 7 kDa. They are abundant in α-helices joined by four intramolecular disulphide bridges. The characteristic feature of this group is a hydrophobic tunnel considered to play a biological role of transferring lipid. However; this activity has not been confirmed. They are stable to peptic digestion, wide pH change and heat denaturing conditions (Brenna et al., 2000). Common examples of allergens are Pru p 3, the major allergen of peach, Cor a 8 from hazelnut and Tri a 14, from wheat (Breiteneder and Radauer, 2004, Hauser et al., 2008). A detailed discussion of this class of protein is given in the following section of this chapter.

1.1.6.2.3 *α-amylase and trypsin inhibitors*

They share common eight stranded conserved α/β barrel structure. These are found in monomeric, dimeric or tetrameric forms with subunits ranging from 120 to 160 amino acids and the same conserved structure characteristic of the superfamily. They can inhibit both α-amylase and proteases

such as trypsin. Cereal α-amylases are important allergens in baker's asthma. Examples of allergens from this group of proteins include Hor v 15 from barley and Sec c 1 from rye (Breiteneder and Radauer, 2004, Hauser et al., 2008).

1.1.6.2.4 *Cereal prolamins*

They are also major storage proteins in cereals. Prolamins from cereals are named according to their source such as gliadins in wheat and secalins in rye. They are found in the endosperm of cereals. The C-terminal region is rich in cysteine and is responsible for intrachain disulphide bonds. The N-terminal is rich in proline and glutamine with repeating pattern of tetra- upto hexapeptides. Common examples reported are Hor v 21 from barley, Sec c 20 from rye and Tri a 19 and Tri a 26 from wheat (Breiteneder and Radauer, 2004, Hauser et al., 2008).

1.1.6.3 Bet v 1 family

A class of plant proteins generally expressed at lower levels in normal conditions, these are induced in plants by infections and abiotic stress. They comprise a group of homologous proteins with an average molecular weight of 17 kDa similar to the first member of the family to be described, Bet v 1, the major birch pollen allergen. More than 95% of birch pollinosis and cross-reactivity with other plant foods is due to this group of proteins. Both, pollen and plant derived allergens of this family share common structural features which bind IgE. They are gathered in the group by their amino acid sequence, serological relationship and enzymic or other biological activity characteristic for the defence. They can be subdivided into 14 classes. However; this classification is expanding by the discoveries of new proteins from varying sources (Edreva, 2005). Examples of the allergens belonging to this group of proteins are Pers a 1 from avocado Cas s 5 from chestnut and Mal d 1 from apple.

1.1.6.4 Thaumatin like proteins

These are 20 kDa proteins consisting anti-parallel β-sheets stabilized by eight intramolecular disulphide bonds very similar to thaumatin, an intensely sweet protein isolated from *Thaumatococcus daniellii*. They are resistant to proteolysis, pH and heat denaturation. They are either naturally occurring antifungal proteins or induced in response to pathogen attack or osmotic stress. Common examples are Mal d 2 from apple, Pru av 2 from cherry and Act d 2 from kiwi (Breiteneder, 2004, Hauser et al., 2008).

1.1.6.5 Profilins

These are low molecular weight proteins ranging from 12 to 15 kDa found in cytosol of all eukaryotes and involved in actin polymerization as they extensively bind actin. The family members are found to be highly conserved in structure and found to be 70% to 85% identical and hence are responsible for high degree of cross-reactivity among the members of this family. They are rich in antiparallel β-sheets. They are found in all organisms thus regarded as pan-allergen. Examples of allergens of this group are Pyr c 4 from pear, Pru av 4 from cherry, Pru p 4 from peach (Breiteneder and Radauer, 2004).

1.1.7 Biological properties of food allergens

Proteins that have the ability to induce allergic response and are present in sufficient quantities to the right context are known as allergens (Breiteneder, 2008). They are usually of variable molecular weight ranging from 9 to 70 kDa. The biochemical properties of allergens may also play a role in determining their allergenicity. Currently we do not understand what the precise characteristics of a protein are, that make it allergen. The abundance of a particular protein in a food is one hypothesis, as most of the allergens reported are the major proteins of the allergenic food. Some of them are glycoproteins, although there are several exceptions like Pru p 3, the major allergen from peach. The stability of the molecule is also a parameter considered to promote its allergenicity. Many allergenic proteins are found to be stable under various harsh conditions like those used in cooking. Similarly gastrointestinal digestion is another major property that may alter the allergenicity since many can survive the harsh denaturing conditions of gastric compartment and usually arrive in the duodenum without being digested or in large stable fragments. The multi-valency of IgE epitopes is another common features of these molecules, a property responsible for the cross-reactivity phenomena found in this group of proteins (Lehrer et al., 2002). For the protein to be allergenic, it must have at least two epitopes each having a length of fifteen amino acids minimum (Huby et al., 2000), which also provides another characteristic feature of allergens which have a molecular weight greater than 3 kDa (being at least 30 amino acids long with two epitopes). Various allergens to date have been characterized as ligand binding proteins, binding metal ions and lipids like fatty acids. The ligand can have a very strong impact on the 3D structure of protein as metal ions are known to affect protein folding to various extents by affecting polypeptide mobilities. The association of the allergens with lipid structures of cell membrane helps them to serve as a defensive force in plants where they may destabilize the cell membrane of infectious microbes,

leading to cell lysis. Lastly, other properties that are found in allergens are the repetitive segments in their structure and aggregation that play very important role in breaking the tolerance (Breiteneder and Mills, 2005a).

1.1.7.1 Epitopes

The specific areas on an antigen which contain immunogenic elements and are recognized by antibodies are known as epitopes (Jerne, 1960). These are specific antigenic determinants on allergens which are identified by IgE in susceptible individuals. According to Benjamin (Benjamin et al., 1984), the whole surface structure of proteins is antigenic, however the recognition of some of the areas on the surface is much quicker than others. Therefore, antigenic proteins have few areas of structure at the surface where they bind to antibodies called epitopes. Generally, these epitopes are found on the surface of native protein and hence, the individual elements of a single epitope may be far from each other in the primary sequence of protein. Due to protein fold and three dimensional structure of protein, these distant amino acids come together and found together on protein surface. Such epitopes are called "conformational" or "discontinuous epitopes". On denaturation of protein, the secondary structure is lost and the individual amino acids of these epitopes get separated from each other resulting in no identification through antibody. Thus recognition through antibody in this case needs a properly folded protein structure (van Milligen et al., 1994, Cooke and Sampson, 1997).

In some cases such as recognition through B-cells, the protein is initially either fragmented due to antigen presenting cell processing or partially unfolded such as in gastrointestinal tract. Still in this case, the protein remains antigenic since recognized by B-cells. In this case, amino acids are found in sequential stretches of 6-12 amino acids on the primary sequence and hence, they retain their immunogenicity even after denaturation. Such epitopes are called "sequential epitopes" (van Milligen et al., 1994, Cooke and Sampson, 1997).

1.2 Plant lipid transfer proteins

Isolation of soluble plant proteins that could transfer phospholipid between membranes *in vitro* began the study of new class of plant proteins called lipid transfer proteins (LTPs) (Kader et al., 1984). They were thought to be involved in trafficking lipids between cellular compartments however, it was later discovered that they are synthesized in premature state containing a signal peptide which directs them into extracellular matrix in the form of mature LTP (Bernhard et al., 1991, Meijer et al., 1993, Thoma et al., 1993). They are widely distributed among plant tissues and

around 13 proteins have been identified as putative or confirmed LTP in *Arabidopsis thaliana* alone (Chae et al., 2009). Despite the unknown function of this protein, they have been reported as antifungal proteins involved in plant defence (Sun et al., 2008). They have also shown important characteristics in food processing such as barley LTP responsible for beer quality and foam formation (Jegou et al., 2000).

LTPs have been reported as important food allergens in fruits, vegetables, nuts, pollen and latex. They have been classed as true food allergens sensitizing through GI tract and are clinically important in Mediterranean areas (Egger et al., 2010, Borghesan et al., 2008).

1.2.1 General properties

LTPs are small (~ 9 kDa for LTP1 or ~ 7 kDa for LTP2) basic proteins with a high pI (~ 9) and share a characteristic eight cystein motif (C . . . C . . . CC . . . CXC . . . C . . . C). They possess a characteristic hydrophobic tunnel like structure enclosed by four α – helices held together by four intramolecular disulphide bridges. A general structure of LTPs is given in Fig. 1.7.

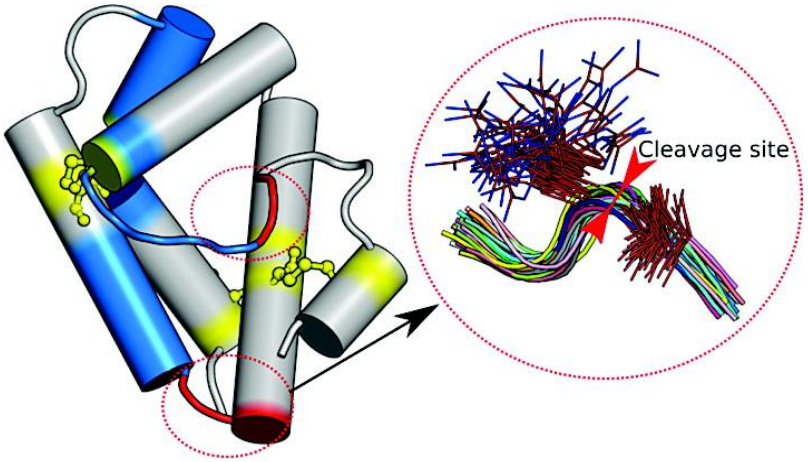


Fig. 1.7: 3-D structure of peach lipid transfer protein. Large solid rods represent helices while small solid rods are loops at C- and N- terminals. The potential cleavage sites are also shown. Reprinted with permission from (Wijesinha-Bettoni et al., 2010).

The first three helices are amphiphilic and parallel to central hydrophobic cavity which can accommodate ligands such as lipid and fatty acids. The cavity is covered at the other end by the fourth helix and loop at C-terminus. There are various residues conserved among this class of protein in addition to the cystines. For example, Tyr 15 and 79, Asp 43 and Arg 44 are well conserved in LTPs (Yeats and Rose, 2008). Molecular dynamic studies have revealed that the

hydrophobic cavity is plastic in nature and expands on ligand binding (Filipp and Sattler, 2007, Lerche et al., 1997, Lerche and Poulsen, 1998). LTPs have shown binding in micromolar concentrations to lipids such as glycerolipids, fatty acids and acyl – CoA with one or two binding sites (Yeats and Rose, 2008).

1.2.2 Localization and biological significance

LTPs have been known to concentrate either in the skins of fruits such as apple and peach or be equally distributed in both the skin and pulp such as apricot and plum. Confocal laser scanning microscopy revealed the location of this protein in the cytoplasm adjacent to the cell wall. Again, the proportion of this protein decreased from pericarp to mesocarp. A very high proportion of LTP was found in the thick wall of the downy hairs of peach peel along with some proportion in the cytoplasm (Borges et al., 2006). Immunocytostaining of peach, apple and plum is given in Fig 1.8 showing localization of respected LTPs. Other locations of LTPs found in cabbage were in the cytoplasm especially in mitochondria, chloroplast and nucleus (Schilling et al., 2003). In case of germinating *Euphorbia lagascae* and sunflower seeds, the LTPs were found to be located within vascular tissues and apoplastic space in the endosperm close to cotyledons (Edqvist, 2003, Pagnussat et al., 2009). In chillies, this class of protein is also found in the intracellular vesicles in lumen (Diz et al., 2011).

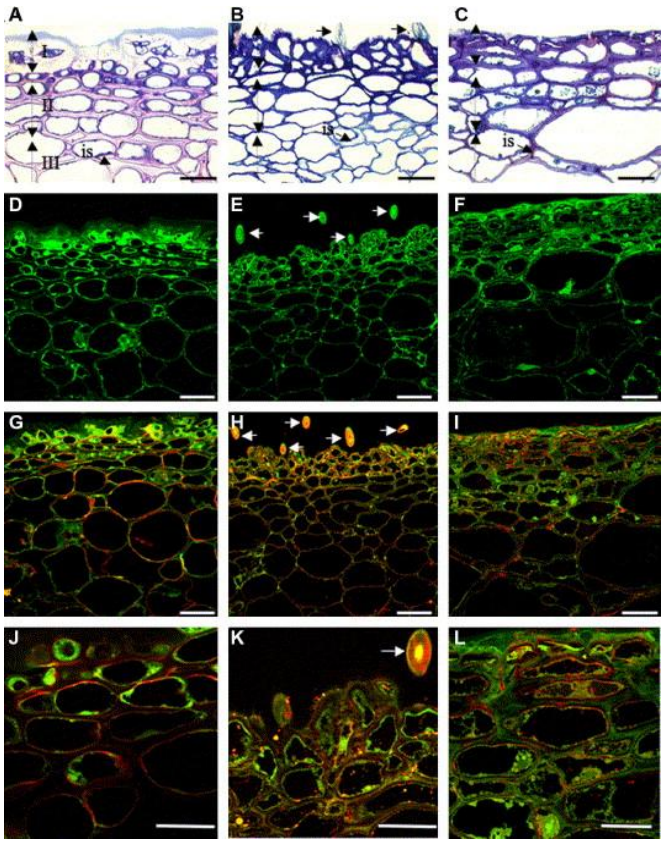


Fig. 1.8: Immunocytostaining of LTPs in apple (A, D, G, K), peach (B, E, H, K) and plum (C, F, I, L). Layers I, II and II represent epidermis, hypodermis and parenhyma respectively. The red colour represents LTP stained. Reprinted with permission from (Borges et al., 2006).

In the context of the biological significance of these proteins, the exact functionality of these proteins remains unclear although several possible roles of this protein in plants have been proposed. They are reported as important in defensive proteins against bacterial and fungal pathogens in radish, barley, *Arabidopsis thaliana*, spinach and onions (Yeats and Rose, 2008). The lipid precursors of cuticle are synthesized in epidermal cell and are transferred through the hydrophobic cell wall, and it has been suggested that LTPs are involved in such transfer activity. They may also be involved in reproductive and vegetative growth of plants such as involved in the extension of cell wall (Yeats and Rose, 2008).

1.2.3 Peach lipid transfer protein

Peach LTP has 91 amino acid residues formed into four helices (H1-H4) connected in a compact domain by four intramolecular disulphide bridges, as for all LTPs. These bridges are formed between H1 and H3 (Cys3 – Cys50), H1 and H2 (Cys13 – Cys27), H2 and H4 (Cys28 – Cys73) and H3 and C-terminus coil (Cys48 – Cys87). The hydrophobic cavity formed here has a polar end

containing Gln35 and Arg44 while a non-polar end containing Leu10, Ala11, Ile14, Leu54 and Val58 (Pasquato et al., 2006). The protein contains eight basic and one acidic residue.

The protein is regarded as an important allergen in the Spanish population, and is the best characterized allergenic LTP, also called Pru p 3 (Salcedo et al., 2008). Originally, it was isolated twelve years ago from the skin of peaches (Pastorello et al., 1999, Sánchez-Monge et al., 1999) and currently; its quantitation by various means such as by using antibodies (Duffort et al., 2002, Carnés et al., 2002) has been well established. The proportion of this LTP was found to be 250 fold higher in peel than the pulp (Ahrazem et al., 2007). The recombinant form of protein is well folded and posses equal biological activity, such as IgE binding capacity, to its natural counterpart (Díaz-Perales et al., 2003). Five conformational IgE binding epitopes have been identified involving Arg39, Thr40, Arg44, Lys80 and Lys91 while three sequential epitopes covering residues 11-25, 31-45 and 71-80 (García-Casado et al., 2003) have also been defined which are homologous with closely related plant LTP allergen epitopes (Borges et al., 2008). T-cell epitopes have also been determined for Pru p 3 and were located between residues 13-27, 34-48 and 43-57, using T-cell cultures from more than 30% of a group of peach allergic patients with 61-75 being the immuno-dominant epitope (Schulten et al., 2009).

1.2.4 Wheat lipid transfer protein

Originally, isolated and sequenced 19 years ago (Désormeaux et al., 1992), wheat LTP was one of the first LTPs to have its 3D structure defined. The protein is 90 amino acid long containing characteristic four helices (Gincel et al., 1994) which are connected by four intramolecular disulphide bridges. The bridges are formed between H1 and H3 (Cys3 – Cys50), H1 and H2 (Cys13 – Cys27), H2 and H4 (Cys28 – Cys73) and H3 and C – terminal coil (Cys48 – Cys87). The two ends of the hydrophobic cavity are exposed to solvent and include both hydrophobic and hydrophilic residues such as Asp7, Arg11, Leu14, Ile54 and Ile58 to one end while His35, Arg44, Pro78 and Val90 on the other end (Charvolin et al., 1999). The protein contains 12 basic and 7 acidic residues.

Wheat LTP, Tri a 14 has been regarded as an important allergen in wheat responsible for bakers' asthma showing 60% sensitization rate as compared to other wheat allergens such as α-amylase inhibitors previously reported as allergens (Palacin et al., 2009, Palacin et al., 2007). The LTP content was found to be equally distributed in bran and endosperm yielding 400 mg LTP1 and 200 mg LTP1b (an adduct of LTP1 with 294 Da compound) from 3 kg of bran (Douliez et al., 2001).

The identification of this LTP has been well established by means of UV, CD, mass spectrometry (Douliez et al., 2001) and immunodetection (Dieryck et al., 1995). The recombinant form of this protein is equally folded and posses equal biological activity in terms of IgE recognition (Palacin et al., 2009).

The conformational epitopes of Tri a 14 have been identified to include several residues namely His35, Asn36, Arg39, Ser40, Asp43, Gly74, Val75, Leu77, Pro78, Tyr79 and Thr80 while sequential epitopes located between 31-40, 51-60 and 66-80. Two of these epitopes share similarity with Pru p 3 i.e residues 31-40 and 66-80 (31-40 and 71-90 in Pru p 3) (Tordesillas et al., 2009).

1.3 Structural stability of lipid transfer proteins

The structural stability of an LTP is thought to be of prime importance in determining its allergenicity as it allows the LTP to retain its 3D structure, thus maintaining conformational as well as sequential epitopes and making available for IgE binding and hence triggering an allergic reaction after exposure. The stability of LTPs relates to their stability during either food processing or digestion in gastrointestinal tract. In some cases, some allergens are denatured during heating and returned to their original conformation after cooling such as Bet v 1 homologues (Gaier et al., 2008). This condition is also referred to as structural stability and may affect the allergenic potential of allergen.

1.3.1 Processing in food context

The term stability refers to the retention of original native 3D structure of LTP after chemical treatment such as urea or physical treatment like heating (Breiteneder and Mills, 2005a). Many LTPs have shown structural stability to heating until 70°C such as peach (Gaier et al., 2008), soybean (Berecz et al., 2010), barley (Perrocheau et al., 2006), cherry (Scheurer et al., 2004) and apple (Sancho et al., 2005). At high temperatures, the behaviour of different LTPs varies depending upon the primary sequence as evident by the fact that some of them denature reversibly while some are irreversible. This behaviour is due to the fact that four intramolecular disulphide bridges hold the helices together with low flexibility (Wijesinha-Bettoni et al., 2010, Murtagh et al., 2003). Pulse electric field and high pressure also did not induce any structural change in apple LTP showing the resistance of LTPs to harsh processing conditions (Johnson et al., 2010). Chemical modifications such as acylation in wheat lipid transfer protein did not show any change in protein secondary structure except hexanoic acid making a slight change in the protein folding (Pato et al., 2002).

Pectin was also found to be protective against heat denaturation of apple LTP (Husband et al., 2011). These examples explain the resistive nature of LTPs to harsh food processing conditions.

1.3.2 GI tract digestion

The resistance to digestion of LTPs allow them to maintain their immunogenic and allergenic motifs and hence, interact with the gut immune system in their active form to sensitize and elicit allergic responses (Salcedo et al., 2004). LTPs have been shown to be resistant to pepsin digestion under physiological conditions *in vitro* (Asero et al., 2000). Many LTPs to date have been shown to have resistance to pepsin digestion *in vitro* such as grapes (Vassilopoulou et al., 2006), cherry (Scheurer et al., 2004), peach and barley (Wijesinha-Bettoni et al., 2010, Cavatorta et al., 2010), wheat (Palacin et al., 2009) and apple (Akkerdaas et al., 2005) except for soybean which was rapidly degraded by pepsin (Astwood et al., 1996). Although LTPs contain Phe and Tyr which are susceptible to pepsinolysis, the flexibility of the target residues in LTPs does not allow such proteolytic interactions making this class of protein pepsin resistant. Under simulated duodenal phase of digestion, LTPs show variable behaviour depending upon again on their primary sequence. For example LTPs from grapes and peaches are digested slowly (Vassilopoulou et al., 2006, Wijesinha-Bettoni et al., 2010) while from barley and wheat remain resistant to digestion (Palacin et al., 2009, Wijesinha-Bettoni et al., 2010). The interaction of physiological components such as phosphatidyl choline also affects the digestibility profile of LTPs such as in grapes, where a protection in duodenal phase of digestion was observed (Vassilopoulou et al., 2006).

1.4 Aims and objectives

The structural stability of LTPs to food processing and gastroduodenal digestion has been well studied in literature and it is proposed that this inherent property of LTPs is important for determining their allergenicity. Such stability is a major factor for this class of proteins to be allergen as hypothesized. The present study was designed to investigate whether these stabilities are important in identifying a protein as potential allergen within a group of similar proteins by taking into account two model LTPs, Pru p 3 and Tri a 14 the former being a more frequent allergen while the later being rare allergen.

The aim of the study was to investigate the behaviour of these proteins under simulated gastroduodenal conditions in the context of the proteins. The behaviour of these proteins was to be determined by incorporating a detailed study on the digestibility of these proteins in the presence and absence of physiological ligands. The affinity to various physiological ligands was to be

determined by ligand binding study through fluorescence mechanisms. The effect of food processing was to be studied by incorporating the thermal processing of the proteins and its effect on the digestibility of denatured proteins. Finally, the effect of food matrix on the structural stability of these LTPs was studied either by expressing Pru p 3 into wheat or through the digestibility of LTPs within their native matrix in both processed and unprocessed forms.

2. Materials and methods

2.1 Biochemical methods

2.1.1 Materials

Cis-Parinaric acid was obtained from Molecular Probes, Invitrogen, Paisley, UK. Bovine serum albumin (BSA -98%), bicinchoninic acid (BCA), copper (II) sulphate (4% w/v) were obtained from Sigma, Dorset, UK. The 96 microtitre well plates were supplied by Inter-med (MaxiSorp^{TM}, Thermo Scientific, Roskilde, Denmark). Sodium chloride was supplied by Sigma, Dorset, UK. Water used throught the study was purified to 18 M Ω cm, using an ELGSTAT UHQ PS unit. Sodium dihydrogen ortho-phosphate was obtained from BDH, Leicestershire, UK. Palmitic acid, 1-Palmitoyl – sn – glycero – 3 – Phosphatidyl Choline (PC), 16-OH Palmitic acid, 12-OH Stearic acid and Linoleic acid were obtained from Sigma, Dorset, UK. 1 mM PC vesicle solution was prepared by dissolving PC into ethanol first and drying under argon over night followed by dissolving the dried PC into 50 mM phosphate buffer pH 7.5 (Mandalari et al., 2009b). Standard precast 4-12% and 12% Bis-Tris gels, LDS sample buffer (4x), MES SDS – running buffer and Coomassie Brilliant Blue were supplied by Invitrogen, Paisley, UK. Methanol, tri fluoroacetic acid (TFA) and acetic acid were supplied by Sigma, Dorset, UK. Acetonitrile was supplied by Fisher Scientific, Loughborough, Leicestershire, UK.

2.1.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.1.2.1 Principles of SDS-PAGE

SDS-PAGE is among the most powerful techniques used commonly for protein analysis. The technique is also used to separate complex mixtures of proteins and can be used to identify the relative molecular mobility of intact protein or separated subunits. The technique may also be used for preparative purpose where a significant quantity of the protein may be obtained for further analysis. When combined with isoelectric focusing (two dimensional electrophoresis), the separation power increases to several fold enabling separation of thousands of proteins from a single mixture (Garfin, 2009). In this study, this technique was used to identify and monitor the homogeneity of proteins during their purification and characterize digestion products of purified proteins as well as proteins from their native plant tissue matrices.

This technique seperates proteins from a mixture depending upon their size, hence gives information about the relative molecular mass of the protein (Mr). Polyacrylamide gels are prepared by polymerizing acrylamide monomers in the presence of bi-functional cross-linking agents such as N',N'-methylenebisacrylamide. The properties of such gels are highly influenced by the degree of crosslinking and pore size (Janson and Rydén, 1998). Therefore, the M_r and R_f are in direct relationship in SDS-PAGE. Proteins to be analyzed are heated in sample buffer in the presence of a suitable reducing agent such as dithiothreitol (DTT) and SDS. The reducing agent (DTT) reduces the disulphide bonds responsible for the tertiary structure of protein whereas SDS denatures and unfolds protein by binding them and creating a uniform rod like shape with a uniformly distributed negative charge. Under the influence of a potential difference, these negative charged protein molecules migrate towards the anode of the electrophoretic chamber and form bands, the sharpness of which depends upon the effectiveness of glycinate ions and proportion of crosslinker in the gel. The migration rate of the proteins through the pores of the gel is relative to their molecular size and charge. For this reason, SDS-PAGE is a simple and reliable tool to determine the molecular weight of proteins (Janson and Rydén, 1998).

2.1.2.2 Methods

SDS-PAGE was carried out in a X-Cell Surelock Electrophoresis system (Invitrogen, Paisley, UK) using a 1 mm thick 4-12% resolving gel. SDS-PAGE samples were prepared by mixing 10 µL 0.5M DTT (Sigma, Dorset, UK) and 25 µL 4x LDS sample buffer (Invitrogen, Paisley, UK) with 65 µL of protein sample. The mixture was heated at 70°C for 10 minutes and applied to precast SDS-PAGE gels (Invitrogen, Paisley, UK) as 5 µg protein per lane for purified proteins. The gel was placed in electrophoresis tank with two compartments, the upper (cathode) and lower (anode) chamber. Both the chambers were filled with running buffer (Invitrogen, Paisley, UK). A constant voltage of 200 V was applied for 35 minutes at ambient temperature with consistent stirring. At the end of the run, the gel cassette was removed from the tank and the gel removed from the cassette using a spatula. The gels were fixed for one hour in fixing solution (40% (v: v) methanol, 10% (v: v) acetic acid in water) followed by a brief washing with water for five minutes. The gels were stained in coomassie brilliant blue Safe – stain (Invitrogen, paisley, UK) for two to three hours followed by destaining in water for one to two hours. The image of the visualized bands was captured using an image scanner (BioRad, Hertfordshire, UK).

2.1.3 Totallab anlysis

Totallab (TL 120, Totallab Ltd., Newcastle upon Tyne, United Kingdom) is a powerful automated software for 1D gel analysis. It sensitively detects the bands in complex patterns within gels and accurately quantifies them which can be further verified through visualization tools. It also calibrates the bands according to the standard molecular weight markers and hence, provides information about the molecular weight of the protein.

The Totallab gel analysis software was used to quantify the band intensities of the intact protein and their digestion products within SDS-PAGE gels. The software uses the file format ".tif" for analysis. Firstly, the image was divided into various lanes by automatic detection tools through selecting the target area on the gel. As the gels retain some background stain, this needs to be removed prior to densitometric analysis. The background removal was made by using program tool "background subtraction" by choosing "rubber band" method which is based on stretching an imaginary rubber band between the edges of first and last band on each lane and considering the pixel counts outside this area as background. After background subtraction, bands were detected by using automatic tool "detect bands" which picks up the variation in number of pixels at various positions within a lane. Profile deconvolution is a process of representing the band pattern of a lane in terms of peaks based on measuring the pixels at various positions of a lane, such that each peak represents a single band. This method then calculates the area under the curves which corresponds to the relative pixel count of the band and hence, represents the volumes of bands through "Gaussian fit" tab. To calculate the molecular weight of the individual bands, R_f calibration was applied where the first band of the standard molecular weight lane is set at $R_f = 0$ while the last band of this lane is at $R_f = 1$. Then the R_f values are matched with the lane containing samples and relative molecular mass calculated.

2.1.4 Ligand binding

2.1.4.1 Principle

The ability of LTPs to bind ligand was studied *in vitro* as means of establishing biological functionality of purified proteins and discovering whether ligand binding affected stability to digestion. There are various binding forces involved in such interactions such as hydrogen bonding, ionic interactions, hydrophobic interactions, cation – π electron interactions and metal complexations (Böhm, 2003).

In order to describe ligand binding reaction, a means of labelling the ligand is required. For example, in many cases, ligand is labelled with a radioactive isotope and the radioactivity of free and bound ligand determined. The interaction of a labelled ligand L* with a protein R may be given as:

$$R + L^* \rightleftharpoons RL^*$$

The concentration of free ligand (L*) at which half of the protein is liganded is known as dissociation constant (K_d). The K_d is half of the maximal binding (B_{max}) (Höfliger and Beck-Sickinger, 2003). The resulting titration curves for these interactions are usually parabolic in nature. In case where the ligand is unlabelled, a suitable labelled ligand can first be bound to proteins and then competed off by displacement with an unlabelled ligand. The concentration, at which half of the labelled ligand is displaced by the unlabelled ligand, is termed as IC_{50}. In this type of interactions, the resulting titration curve is usually sigmoidal (Höfliger and Beck-Sickinger, 2003). Usually, the IC_{50} values depend upon the concentration of labelled ligand and vary between experiments. To overcome this problem, another constant is defined by Cheng (Cheng, 2002) called K_i which is independent of labelled ligand concentration . This constant is defined as:

$$Ki = \frac{IC_{50}}{1 + \frac{[s]}{K_d}}$$

Where [s] is the concentration of labelled ligand.

Molecules that fluoresce undergo excitation and emission with respect to their electronic energy level when exposed to light of a particular wave length. The interaction of a fluorescent probe with its environment affects its fluorescence parameters such as absorption maxima (λmax), quantum yield and lifetime (Moller and Denicola, 2002). In general, by decreasing the polarity of the surrounding medium, the fluorescence of a probe may increase or decrease such as in the case of binding to the hydrophobic sites of a typical protein (Moller and Denicola, 2002) including ligand binding tunnel.

Cis-Parinaric acid (CPA) is a conjugated polyene 18C fatty acid with four unsaturated bonds in the middle of the hydrophobic chain making it minimaly hydrophobic and can be used as a fluorescent probe in ligand binding studies (Fig. 2.1).

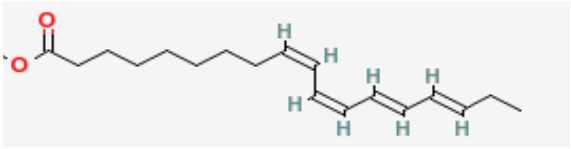


Fig. 2.1: Chemical structure of CPA showing the position of highly conjugated double bonds.

Whilst the fluorescence emission spectrum of cis-parinaric acid (CPA) is unaffected by its environment, the quantum yield is sensitive to solvent, increasing to the same degree as the solvent polarity of the solvent changes (Sklar et al., 1977a). In aqueous systems, CPA has a low quantum yield, which increases several fold when bound to hydrophobic sites of a protein (Sklar et al., 1977b). Consequently, the absorbance maximum (λ_{max}) of CPA shifts after binding with a protein such as human peroxisome proliferator activated receptor γ (Palmer and Wolf, 1998). These characteristics make it suitable to probe ligand binding of proteins containing hydrophobic domains.

To compare the structural behaviour of these proteins for adopting ligands from the food matrix or during the digestion, a model study was carried out by incorporating some physiological ligands. For binding of physiologically relevant ligands, two groups of ligands were selected. In first group, ligands were selected which were relevant to plants and included palmitic acid, linoleic acid, 16-OH Palmitic acid and 12-OH stearic acid. The structures of these ligands are given in Fig. 2.2.

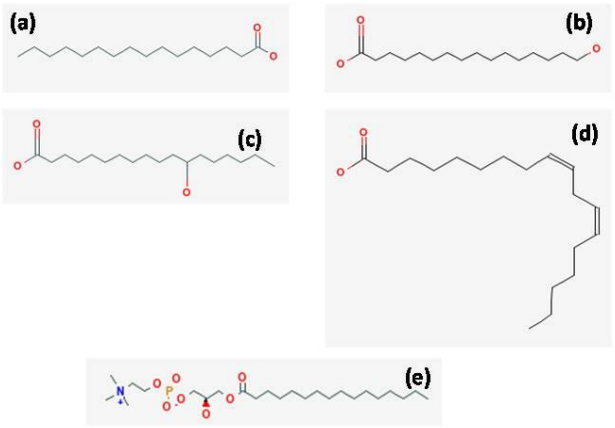


Fig 2.2: Chemical structures of ligands (a) Palmitic acid, (b) 16-OH Palmitic acid, (c) 12-OH Stearic acid, (d) Linoleic acid, (e) 1-Palmitoyl – sn – Glycero – 3 – Phosphocholine.

In the other group, comprising biosurfectants found in digestive juices included lysophosphatidylcholine (1-palmitoyl – sn – glycerol – 3 - phosphatidyl choline), bile salts corresponding to sodium glycodeoxy cholate and sodium taurocholate and lipopolysaccharide.

Lysophosphatidyl choline used here was in two forms, monomeric form and micellar form (vesicles). Palmitic acid and linoleic acid are the major fatty acids in peach (Izzo et al., 1995) and wheat (Morrison, 1978). 16-OH palmitic acid is a component of synthetic cutin (Irwin et al., 1998) while phosphatidyl choline is secreted in oral and gastric mucosa during digestion (Dial et al., 2008, Bernhard et al., 1995). The bile salts are important components in the physiology of lipid digestion and are secreted during the digestion (Hofmann and Small, 1967). Lipopolysaccharide (LPS) are the found in the outer membrane of gram negative bacteria and hence are the part of microbial flora of food and digestive system (Gronow and Brade, 2001).

2.1.4.2 Methods

2.1.4.2.1 Ligand binding at pH 7.5

2.1.4.2.1.1 Binding of cis-parinaric acid to LTPs

Two different methods were employed here. The first method was based on the method described by Palmer (Palmer and Wolf, 1998). Absorbance was measured using a Lambda 35 UV/Vis Spectrophotometer (Perkin Elmer, Cambridge, UK). In this method, protein at 5 µM concentration was mixed with 3 µM concentration of CPA in 100 mM phosphate buffer pH 7.5. The aborbance of this solution was scanned in the range of 312-340 ηm. Blank and CPA alone were also scanned in the same range to compare the shift in absorption maximum. The second method was established on the basis of reported method by Cooper et al (Cooper et al., 2002). Fluorescence intensity was measured at 25 °C using an LS55 Luminescence Spectrometer (Perkin Elmer, Cambridge, UK), which had doubts in its functionality during the data collection as determined by measuring the fluorescence of a solution repeately along with the standards. The instrument was turned on at least one hour ago before taking measurements to warm up and stabilize the lamp. The slit width was adjusted to 5 mm for both excitation and emission. The measurement was taken for no longer than 1.5 – 2 seconds to avoid destruction of CPA which in turn, reduces the fluorescence intensity measured. CPA was prepared as a 3 mM stock solution in ethanol, the concentration being determined spectrophotometrically using an extinction coefficient of ε_{308} = 7.9 x 10^4 $M^{-1}cm^{-1}$. Further dilutions of this probe were made in degassed ethanol. The excitation and emission wavelengths were determined by scanning a 10 µM CPA solution in 50 mM phosphate buffer pH 7.5 and were found at 320 nm and 420 nm respectively. Samples were handled carefully avoiding light to prevent degradation of CPA. All the protein solutions were prepared in 50 mM phosphate buffer pH 7.5. Beta lacto globulin (BLG) was used as a test protein and positive control in the

development of the assay. One millilitre of protein solution was poured into a cuvette and titration was performed by adding 1 µL injections of CPA in ethanol in a stepwise manner. The fluorescence intensity recorded at various injections was plotted against the total concentration of CPA within the cell. In case of BLG, the observed fluorescence was very high. Therefore, a lower concentration of protein ranging from 500 nM -1 µM protein was used. For LTPs, the concentration used within the cell was 5 µM.

2.1.4.2.1.2 Binding of non-fluorescent ligands to LTPs

To explore the binding of other ligands which were not fluorescent, a displacement assay was used. One millilitre of 5 µM protein solution containing CPA at or below its *Ki* was poured into the cuvette and allowed to equilibrate for 2-3 minutes with gentle mixing. Five ligands were used including palmitic acid, 16-OH palmitic acid, 12-OH stearic acid, linoleic acid and 1-palmitoyl – sn – glycerol – 3 – phosphatadyl choline (PC). PC was used in its native as well as vesicular form (Mandalari et al., 2009b). The displacing ligand solutions were prepared at a concentration of 1 mM and were injected into the cuvette in 1 µL aliquots.

2.1.4.2.2 Ligand binding under gastric conditions

The binding of ligand with LTPs was also observed under gastric conditions. Protein was dissolved in simulated gastric fluid (SGF: 150 mM NaCl pH 2.5) and the pH was adjusted to 2.5. One millilitre of the protein solution was poured into the cuvette and titration was carried out with CPA or other physiological ligands in the same way as described above. PC vesicles were prepared in the same way as described in section 2.1.1 with a modification that dried PC was dissolved in SGF.

2.1.5 Protein determination

Protein quantification was carried out using a BCA Assay. The structure of BCA and the principle of BCA assay is given in Fig 2.3.

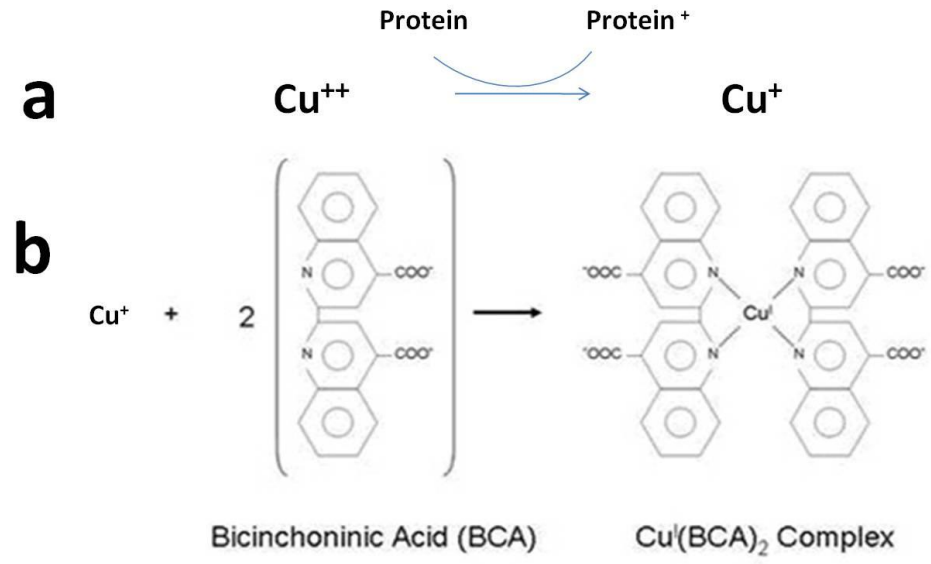


Fig. 2.3: Chemical structure of BCA complex (reprinted by permission from (Brescia and Banks, 2010, Brenner and Harris, 1995)). Reduction of Cu^{++} into Cu^{+} by protein (a) and subsequent complexion of Cu^{+} with BCA (b).

The assay is based on the measurement of the Cu^{+} by using Bicinchoninic acid which is formed by the conversion of Cu^{2+} into Cu^{+} by proteins under alkaline conditions forming Biuret complex. The Biuret complex is formed by cysteine, cystine, tryptophan and tyrosine residues of the protein with Cu^{++} which is reduced to Cu^{+} in the complex in alkaline environement depending upon the temperature. The Cu^{+} then reacts with BCA forming an intensed purple colour complex showing absorbance maximum at 562 nm. At elevated temperature such as 37°C to 60°C, a persistent complex is formed by Bicinchoninic acid by chelating Cu^{+} ions formed which is directly proportional to the quantity of protein. This complex shows strong absorbance at 562 nm. The sensitivity is high with low variation at higher temperatures. The quantity of protein is measured spectrophotometrically along with known standards (Smith et al., 1985). The advantages associated with the assay are the stability of complex and tolerance to the interfering substances such as detergents (Walker, 2002).

The BCA assay was performed with BCA assay kit (Sigma, Dorset, UK). Protein standards were prepared by making a stock solution of BSA at 10 mg/ mL and diluting it to provide 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ mL standards. The BCA working reagent was prepared by mixing 50 parts of reagent A with 1 part of reagent B which gave a green coloured solution. The reagent was prepared just before the use. Samples and standard in 25 µL volume were mixed with 200 µL of BCA reagent in triplicate in a 96 well microtitre plate. The mixture was incubated at 37°C for 30 minutes with shaking to ensure uniform distribution of the reagents. At the end of incubation, samples

containing protein became purple in colour whose intensity was measured by reading the plate in a plate reader (x-Mark microplate absorbance spectrophotometer, BioRad, Hertfordshire, UK) at 562 nm. Protein quantity was determined by comparing the reading with those of standards by applying a calibration curve. An example of a calibration curve is given in Fig. 2.4.

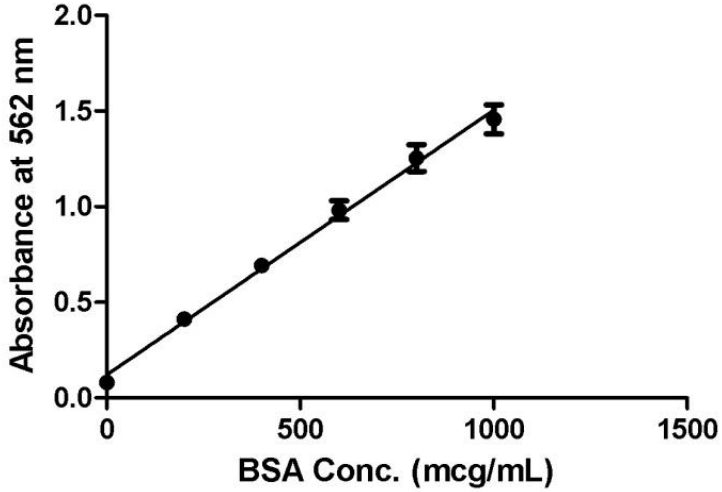


Fig. 2.4: An example of the calibration curve developed to determine protein concentration in samples.

2.1.6 CD spectroscopy

Far UV – Circular dichroism (CD) is a valuable spectroscopic technique for studying protein secondary structure in solution. It measures the differences in absorption between left-handed and right-handed polarized light. The differences in absorptions are due to the asymmetric nature of the chiral groups in protein molecules. The protein sample is scanned over a range of wavelength covering both the far (178-250 nm) and near – UV (250-300 nm) region of the spectrum. Far – UV CD spectra of proteins generally reflects the secondary structure of proteins since the structural components of protein have characteristic far-UV CD spectra. α- helices show two negative peaks (negative ellipticity) at 208 and 222 nm with a positive peak (positive ellipticity) at 193 nm. β-sheets exhibit a broad negative band near 218 nm and a large positive band near 195 nm. The disordered extended chains have a weak broad positive band near 217 nm and a large negative band near 200 nm. CD spectroscopy may also be used to examine tertiary structures of proteins because the chromophores of aromatic amino acids show distinct CD spectrum in the near UV region due to the dissymmetric environment (Greenfield, 1999). The data is analyzed by comparing it with reference protein sets containing 15 to 33 proteins, some of which are α-rich structures, some are β-rich structures while some are αβ-rich structured proteins. Sometimes, a reference set of denatured proteins is also used to compare. Therefore, protein secondary structure determination is limited to

the reference sets only (Sreerama and Woody, 2000). CD-Pro is a free for scientific research available program to analyze the CD data with analysis programs such as CDSSTR and ContinLL which use several reference sets of data called IBasis ranging from 1-7 with known secondary structures of proteins. The analysis program compares the ellipticity of the sample protein to ellipticities of proteins with known secondary structures available in the data set.

Circular dichroism (CD) of the protein solution was measured in 20 mM phosphate buffer pH 7.0 and spectra were recorded from 270 – 190 nm with J-710 spectropolarimeter (Jasco Corp. Tokyo, Japan) against the same buffer by using a 0.1 mm path length quartz cell. Spectra represents the average of four accumulations collected at 20 nm/ min with a 4 sec. time constant, a 0.5 nm resolution and sensitivity ±100 mdeg. The data was analyzed by using CDPro. Two different programs were used in the software to analyze the data namely CDSSTR and ContinLL. Each program utilizes seven different sets of reference spectra to match with, namely IBasis 1 to IBasis 7.

2.2 Digestion methods

2.2.1 Materials

All the chemicals used were of analytical grade. Sodium chloride, trypsin, chymotrypsin, Bowman – Birk trypsin chymotrypsin inhibitor, methanol, acetic acid and ammonium bicarbonate were obtained from Sigma, Dorset, UK. LDS sample buffer (4x), MES SDS-running buffer (20x), Coomassie brilliant blue Safe – stain and 4-12% standard precast SDS-gels were obtained from Invitrogen, Paisley, UK.

2.2.2 Simulated gastroduodenal digestion

The gastrointestinal tract is composed of several organs and glands which play important roles during digestion and absorption of foods. Depending uon the time, amount, composition and physicochemistry of food, the digestion results in breakdown of food structure by enzymes secreted such as splitting of proteins, carbohydrates and fats with the help of muscle contraction which promotes digestion by physical mobilities of secretions into the food (Coupe et al., 1991, Hasler, 2009). *In vitro* models have been developed to mimic the behaviour of foods or components of it into *in vivo* system. In such models, characteristics of reactants such as enzyme activity, ionic strength and composition, mechanical mimicking, digestion times and type of sample affect the out put of these processes (Boisen and Eggum, 1991). Predominantly proteases found in GI tract are pepsin (found in stomach) and trypsin/ chymotrypsin (found in small intestine). Other enzymes

include lipases (gastric and pancreatic lipases) and amylases (salivary and duodenal α-amylases) (Hur et al., 2011).

The lipases found during digestion absorb at the surface of emulsified lipid and hydrolyze triglycerides into diglycerides, monoglycerides and fatty acids. The activity of pancreatic lipase depends upon co-lipase. The digestion products thus produced are absorbed into mixed micelles and transported to epithelium cells through mucus layer. Short chain fatty acids absorb directly while long chain fatty acids first form a micelle with bile salts and lecithin which absorb at luminal cell surface (Ratnayake and Galli, 2009). Amylases convert mainly starch into oligosaccharides and monosaccharides. Most common amylase used in *in vitro* models is α-amylase (Koh et al., 2009).

Pepsin is predominantly found in gastric compartment, released as pepsinogen which is denatured by HCl in stomach and auto-cleaved to form active intact pepsin. The active site of pepsin consists Asp-Thr-Gly, which is the hallmark of aspartic proteases (Davies, 1990). Pepsin cleaves peptides bonds between hydrophobic and aromatic amino acids (Dunn, 2001). Trypsin is produced in pancreas as trypsinogen, converted into mature trypsin by enteropeptidase and released in duodenum. It hydrolyses C-terminus bonds of Lys and Arg except Pro is followed by these amino acids (Leiros et al., 2004). Chymotrypsin is also produced in pancreas as chymotrypsinogen which is activated by tryptic hydrolysis followed by autolysis into maturechymotrypsin. It hydrolyses C-terminus of Tyr, Phe and Trp (Hedstrom et al., 1992).

2.2.2.1 Digestion of purified proteins

2.2.2.1.1 Simulated gastric digestion

Purified proteins (5 mg) were dissolved in 9 mL of simulated gastric fluid (SGF: 150 mM NaCl pH 2.5) and the pH was returned to 2.5 by using 1M HCl in a clean sterilin bottle. The final volume was made up to 9.9 mL with SGF. The solution was kept for one hour in a shaking incubator (New Brunswich Scientific, Fischer Scientific, Loughborough, Leicestershire, UK) to reach the digestion conditions (170 RPM, 37°C). A volume of 100 µL of freshly prepared pepsin solution in chilled SGF was added into the protein solution such that pepsin to protein ratio was maintained as 1:20 (w/w). The digestion was carried out for one hour with samples (100 µL) drawn at various time points one after adding enzyme (20 sec) and at time points 1, 2, 5, 10, 20, 40 and 60 minutes. The digestion was stopped in 100 µL samples by the addition of 20 µL of 0.5 M ammonium bicarbonate solution which raised the pH of the mixture above 7 inhibiting pepsin activity. All the samples

taken were kept in ice during the remainder of the experiment and then stored at -20°C until analyzed.

2.2.2.1.2 Simulated duodenal digestion

At the end of gastric digestion phase, the pH of the remaining digestion solution was raised up to 6.5 by dropwise addition of 1M NaOH to avoid sharp increase in the pH and prevent protein denaturation. A volume of 5 mL of this gastric digest was transferred into a clean sterilin bottle for duodenal digestion and mixed with 275 µL of 0.5 M BisTris pH 6.5 in simulated duodenal fluid (SDF: 150 mM NaCl, pH 6.5) and 125 µL of bile salt stock solution (186 mM sodium glycodeoxycholate, 142.65 mM sodium taurocholate dissolved together in SDF). The mixture was equilibrated at 37°C with shaking at 170 RPM for 10 minutes prior to adding 50 µL of each of trypsin (13,000-20,000 units/ mg determined by BAEE (N-benzoyl, L-arginine ethyl ester) as substrate) and chymotrypsin (40 units/ mg protein determined by BTEE (N-benzoyl L-tyrosine ethyl ester) as substrate) solution freshly prepared to maintain the proportion of 1:4:400 (w: w: w) to protein respectively. Samples were withdrawn at time points 20 sec, 1, 2, 5, 10, 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes. Digestion was stopped by adding 10 µL of 0.5 mg/mL Bowman Birk trypsin/ chymotrypsin inhibitor (1 mg inhibiting 0.5 mg trypsin or 1 mg chymotrypsin with the same activities as used in this protocol) to 100 µL of the digest. Bowman – Birk inhibitor forms a ternary complex with the active sites of trypsin and chymotrypsin making them unavailable for reactivity with substrate. The samples collected were analyzed by SDS-PAGE and MALDI – ToF – MS and Orbitrap MS.

2.2.2.2 Digestion of foods

2.2.2.2.1 Model chew

A “chew” was prepared by using a mincer (Lakeland, Cumbria, UK) to mimic the oral chewed material. Peach peel was separated from the fruit by using a domestic knife such that the thickness of the peel was kept 2-3 mm. Peel was removed and subjected to the model chew immediately after the removal. The chew was subjected to digestion experiments immediately after preparation. The mincer and chew are given in Fig. 2.5.

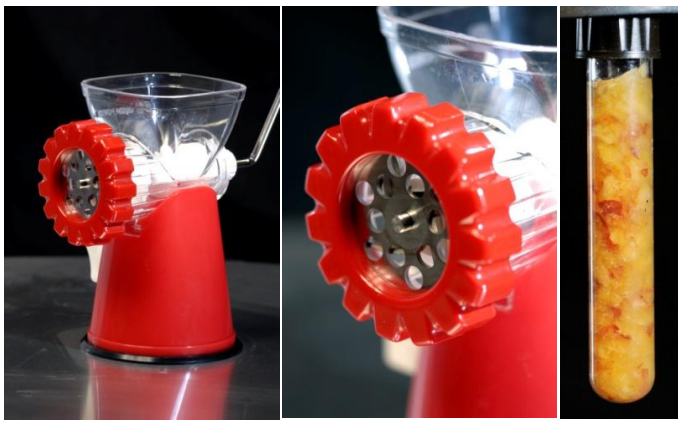


Fig.2.5: Mincer (left) showing the disc (middle) and model chew of peach peel (right).

In case of bread digestion, the crust and crumb were separated from the bread manually and chewed in the same way as for peach peel.

One gram of model "chewed" peach peel was mixed with 600 µL of model saliva (150 mM NaCl, 3 mM urea, and human salivary amylase 2U / mL pH 6.9). The digestion was carried out at 37°C with shaking at 170 RPM for two minutes and stopped by dropping the pH to 2.5 using 1 M HCl. In the case of raw flour or "chewed" bread, 200 mg of this material was mixed with 600 µL of model saliva and incubated for two minutes at 37°C with shaking at 170 RPM. The digestion was stopped by dropping the pH to 2.5 using 1 M HCl.

2.2.2.2.2 Preparation of PC vesicle

2.2.2.2.2.1 Gastric PC

Commercially available egg lecithin (Avanti Polar Lipids, Alabama, United States) dissolved in chloroform (63.5 mM) was dried under argon and vacuum dried over night in a round bottomed flask. SGF was added to the dried PC to make a final concentration of 0.5 mM and sonicated to clarity. This solution was always used fresh.

2.2.2.2.2.2 Hepatic mix

Egg lecithin and cholesterol solution prepared individually in chloroform were dried under argon and vacuum dried overnight in a round bottomed flask. SDF was added to this dried mixture to a final concentration of 6.5 mM PC and 3 mM cholesterol. To this solution, bile salts were added to a final concentration of 12.5 mM sodium taurocholate and 10.5 mM sodium glycodeoxycholate. The solution was sonicated to clarity and was freshly used.

2.2.2.2.3 Simulated gastric digestion

In case of digestion of food matrix, oral digests were adjusted to pH 2.5 using 1 M HCl. The volume of added fluid was made up with simulated gastric fluid (SGF, 150 mM NaCl pH 2.5) to 1250 µL. A volume of 550 µL of gastric PC solution was added to the sample and kept at 37°C with shaking at 170 RPM for 10 minutes to reach the digestion temperature. A volume of 100 µL of freshly prepared pepsin solution in chilled SGF was added into the digestion mix to maintain the pepsin to protein ratio as 1:20 (w: w) respectively. Samples were removed after 0, 1, 2, 5, 10, 20, 40, 60 and 120 minutes. The digestion was stopped by adding 250 µL of 0.5M ammonium bicarbonate solution which raised the pH above 7.0. The digests were stored at -20°C until required.

2.2.2.2.4 Simulated duodenal digestion

At the end of gastric digestion, the pH was increased to 6.5 by using 1 M NaOH. The digest was mixed with 60 µL of 0.5 M BisTris pH 6.5 and 340 µL of hepatic mix (section 2.2.4.2.2). Pancreatic lipase (3.98 µL, 25 U/ µL), co-lipase (16 µL, 0.55 mg/ mL) and pancreatic amylase (12.7 µL, 4 U/ µL) were added just before adding the proteases. Freshly prepared trypsin (5 µL) and chymotrypsin (20 µL) were added into the digestion mix to maintain the ratio of 1:4:400 (w: w: w) to protein respectively. The digestion was stopped at 0, 1, 2, 5, 10, 15, 30, 60, 90 and 120 minutes by adding 125 µL of Bowman Birk trypsin-chymotrypsin inhibitor (15 mg/ mL). The samples were kept in ice during the digestion and stored at -20 °C until required.

2.3 Mass spectrometry

Mass spectrometry has become a method of choice for studying complex protein samples. This is an analytical method that provides qualitative and quantitative information about proteins. The fundamental principle behind this technique is based on the fact that charged protein or peptide molecules migrate in a vacuum under the influence of electric or magnetic fields in a manner dependant upon their mass to charge ratio.

There are three main fundamental parts in a mass spectrometer; an ion source, a mass analyzer and a detector. The ion source generates analyte molecules in ionic form. Protein molecules are first ionized by suitable means such as fast atom bombardment (FAB), electron impact (EI) or chemical ionization (CI). These ions then migrate through the mass analyzer. The mobility of these charged analytes dependent on the mass and charge. The mass analyzer then measures the mass to charge

ratio of ionized analytes. The arrival of these analytes at the detector results in a specific signal depending upon the m/z of the analyte. The intensity of the signal reflects the population arriving at the detector. The detector registers the number of ions at each m/z value (Ho et al., 2003, Aebersold and Mann, 2003).

2.3.1 Materials

Sinapinic acid, trifluoroacetic acid, dithiothreitol (DTT), betalactoglobulin (BLG) was of 98% purity while acetonitrile was of analytical grade and both were obtained from Sigma, Dorset, UK. Myoglobin (95-100% purity) was used for calibration of the MS spectra and was obtained from Sigma, Dorset, UK. Standard MALDI target plates were obtained from Bruker Daltonics, Coventry, UK.

2.3.2 In gel trypsin digestion sample preparation for MS

To identify a protein, samples are excised from the gel or a solution of protein is directly digested by a suitable specific enzyme with predictable cleavage such as trypsin which reliably cleaves on the carbonyl side of lysine and arginine under optimal conditions. For MS/MS analysis, samples were digested in – gel with trypsin and identified by searching with MASCOT (Perkins et al., 1999). The in gel tryptic digestion was performed by Fran Mulholland of the JIC/ IFR joint Proteomics Partnership. The gel spot was excised and transferred to a PCR tube. Destaining was carried out by adding 100 µL of 20% (v: v) acetonitrile, 80% (v: v) 50 mM ammonium bicarbonate to the gel for 20 minutes. Dehydration of the gel slices was carried out by adding 100 µL of acetonitrile for 20 minutes. The rehydration and in gel digestion was carried out by adding 5 µL of 10 mM ammonium bicarbonate containing 50 ng of sequencing grade trypsin (Sigma, Dorset, UK). The digestion was performed for 3 hours at 37˚C and the digestion was stopped by adding 5 µL of 5% (v: v) formic acid creating also a +ve charge on protein. The MS spectrum was recorded with Micromass Q-ToF II fitted with Waters CapLC System. The sample was sprayed through the tip of a borosilicate nano spray needle held at a potential of +800 V into the source of mass spectrometer. Mass spectra were acquired over the range of 50-1600 m/z. The most intense signals were selected for collision induced defragmentation.

For MALDI – ToF – MS, samples were prepared either reduced by DTT or used unreduced. A volume of 105 µL of protein solution was mixed with 150 µL of digestion buffer (50 mM ammonium bicarbonate) and 15 µL of either 100 or 500 mM DTT for a final concentration of 5 or

25 mM. The solution was vortex mixed and heated at 95°C for either 5 or 30 minutes. After reduction, alkylation was carried out by adding 30 µL of either 100 or 500 mM iodoacetamide for a final concentration of 10 or 50 mM. The solution was kept in the dark for 20 minutes. The sample is then applied to MALDI – ToF – MS target plates together with matrix solution. Most common matrices used for proteins and peptides are 3,5-dimethoxy-4-hydroxybenzoic acid (sinapinic acid), α-cyano-4-hydroxy-cinnamic acid (CHCA) and 2,5-dihydroxy benzoic acid (DHB) (Gross, 2004). In the case of MS of simulated digestion products, the samples are diluted to 1 µg/ mL in 0.1 % (v: v) TFA and either directly spotted onto target plates or applied to orbitrap MS. The MALDI – ToF – MS of the samples was performed according to the method described (Wijesinha-Bettoni et al., 2010). Briefly, the MALDI matrix was prepared by suspending sinapinic acid in 30% (v: v) acetonitrile and 0.1% (v: v) TFA to saturating concentration. The suspension was sonicated for 10 minutes for optimal solubility. A small volume (0.5 µL) of this matrix solution was spotted on a standard polished stainless steel plate (Bruker Daltonics, Coventry, UK) followed by applying the same volume of sample (0.5 µL) onto the matrix solution. The spot was air dried prior to MALDI – ToF – MS analysis. Alternatively, a Prespotted AnchorChip (PAC, Bruker Daltonics, Coventry, UK) plate already spotted with MALDI matrix was used for this analysis.

2.3.3 MALDI – ToF – MS

The matrix assisted laser desorption ionization – time of flight – mass spectrometry (MALDI – ToF – MS) is a technique based on laser desorption/ ionization which was originally introduced in late 1960s (Vastola et al., 1970, Vastola and Pirone, 1968, Fenner and Daly, 1966). It utilizes a solid sample layer on which laser light (ranging from UV to IR) is absorbed generating evaporated sample. A basic MALDI – ToF – MS instrument is given in Fig 2.6.

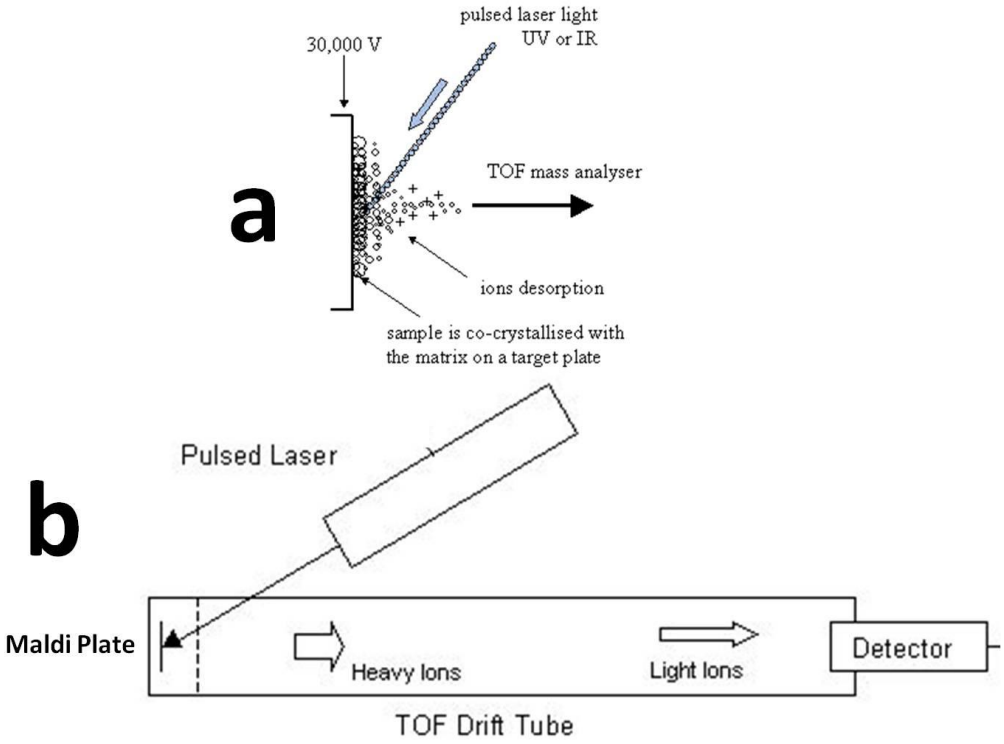


Fig. 2.6: Schematic representation of ionization of analytes at MALDI plate (a) and subsequent identification through the detector (b).

When laser light is applied to the spot, energy is transferred to the matrix raising the temperature which results in evaporation of the peptides carrying charges. The ions thus are aligned by using a focussing lens. These ions then accelerate under the influence of voltage towards a time of flight (ToF) mass analyzer. ToF mass analyzer may be linear or reflectron which provide varying sensitivity and can detect proteins with molecular weight of upto 500 kDa. The ions migrate within the mass analyzer in various time lengths according to their charge to mass ratio (m/z). The detector set at the end of the analyzer then records the population of the ions arriving at certain time and creates a spectrum representing all the events occurred at the detector in on shot. The precision in such cases may be around 10 ppm.

The MS of the samples was carried out by Phil Johnson at IFR. The MALDI plate prespotted with sample was loaded to Bruker Ultraflex MALDI – TOF/TOF mass spectrometer (Bruker Daltonics, Coventry, UK) equipped with a pulsed N_2 laser (λ = 337 nm, frequency 10 Hz). Protein spectra were recorded in two different ranges, small molecular weight or peptide range (0-4000 Da) and high molecular weight or protein range (2000-12000 Da) in linear mode at an accelerating voltage of 25 kV by averaging of atleast 300 individual laser shots. Myoglobin was used as the internal standard to calibrate the instrument.

2.3.4 Orbitrap MS

The MS of samples was carried out by Phil Johnson at IFR. For analysis of the digests of LTPs, a solution of gastroduodenal digests of LTPs was adjusted at 1 µg/ mL concentration in 0.1 % (v: v) TFA. RP-HPLC-ESI-MS was performed using an Accela HPLC coupled to an Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, USA). A volume of 25 µl of sample preparaed was applied to the reverse phase HPLC column (Phenomenex Jupiter 5 µm C4 150 x 2 mm i.d.). The column was washed for 10 minutes with 5% (v: v) acetonitrile. The elution was carried out with a linear gradient of 5-50% (v: v) acetonitrile over 50 minutes. The eluate was directly applied to orbitrap and MS obtained in positive ion electrospray mode. MS/MS spectra were obtained using collision induced dissociation (CID) with collision voltage 35 V. The spectrum was recorded between a mass range of was *m/z* 300 – 2000.

2.3.5 Bioinformatic methods

Bioinformatics is the study of the biological problems such as analyzing protein to understand its structural characteristics by using available computational and statistical methods. In most of the definitions, it is narrowed to computational molecular biology only (Counsell, 2003). Its primary use has been made in genomics and genetics where it has been applied mainly in the sequencing of large scale DNA but is increasingly being used for analysis of metabolomic and proteomic data sets. The primary objective of this technique is to increase understanding of biological processes by focussing on computational methods. In our study, the bioinformatic methods are used to analyze proteomic data.

2.3.5.1 MASCOT database searching

MASCOT is a powerful database search engine which uses the data obtained after mass spectrometry for protein identification from available primary sequence databases of proteins. There are two steps of identification namely peptide mass fingerprinting (PMF) and MS/MS. In PMF, MS data resulting from digestion of a protein with endo proteases such as trypsin are used to search existing sequence database. The experimental mass values of the peptides obtained by mass spectrometry are matched with the calculated mass values of the theoretical fragments of the candidate protein digested in silico by the same enzyme using defined cleavage rules. In case of MS/MS, the peptides are fragmented under vacuum by using an inert gas and the masses of the

fragments measured. These masses of the fragments are then matched to the theoretical masses of the fragments of the same peptide. On the basis of scoring algorithm, the closest match is identified.

The spectrum was analyzed for "peptide mass mapping" or "peptide mass fingerprinting" by submitting the data for database searching using the Matrix Science Mascot search engine. The MOWSE (MOlecular Weight Search Engine) score of 70 or more was regarded as significant. The schematic representation of the process is given in Fig. 2.7.

2.3.5.2 MMass analysis

MMass is a flexible tool released under general public license for analysis and interpretation of mass spectrometric data. It offers a variety of data processing tasks and simulated experiments in-silico. It offers other analysis tools such as manipulating the spectrum, data processing and calibrating. It also offers in-silico simulated experiment tools related to protein sequence such as sequence editing, theoretical protein digestion, peptide fragmentation etc. The mass calculator calculates the masses of the peptides, fragments along with the composition and isotopic patterns (Strohalm et al., 2010). The peptides obtained after simulated digestion of LTPs were analyzed by MALDI – ToF – MS and the intact mass of the peptides obtained. The sequence of LTPs as identified was obtained from SwissProt Database and was digested in-silico with MMass with combined trypsin/chymotrypsin cleavage rules both under non-reducing and reduced alkylated conditions. The theoretical masses obtained for peptides were then matched with experimental masses obtained under simulated digestion. The masses matched were then identified as the target peptide as a result of digestion. Together with identified peptides, there were other unidentified peptides found in the experimental mass list.

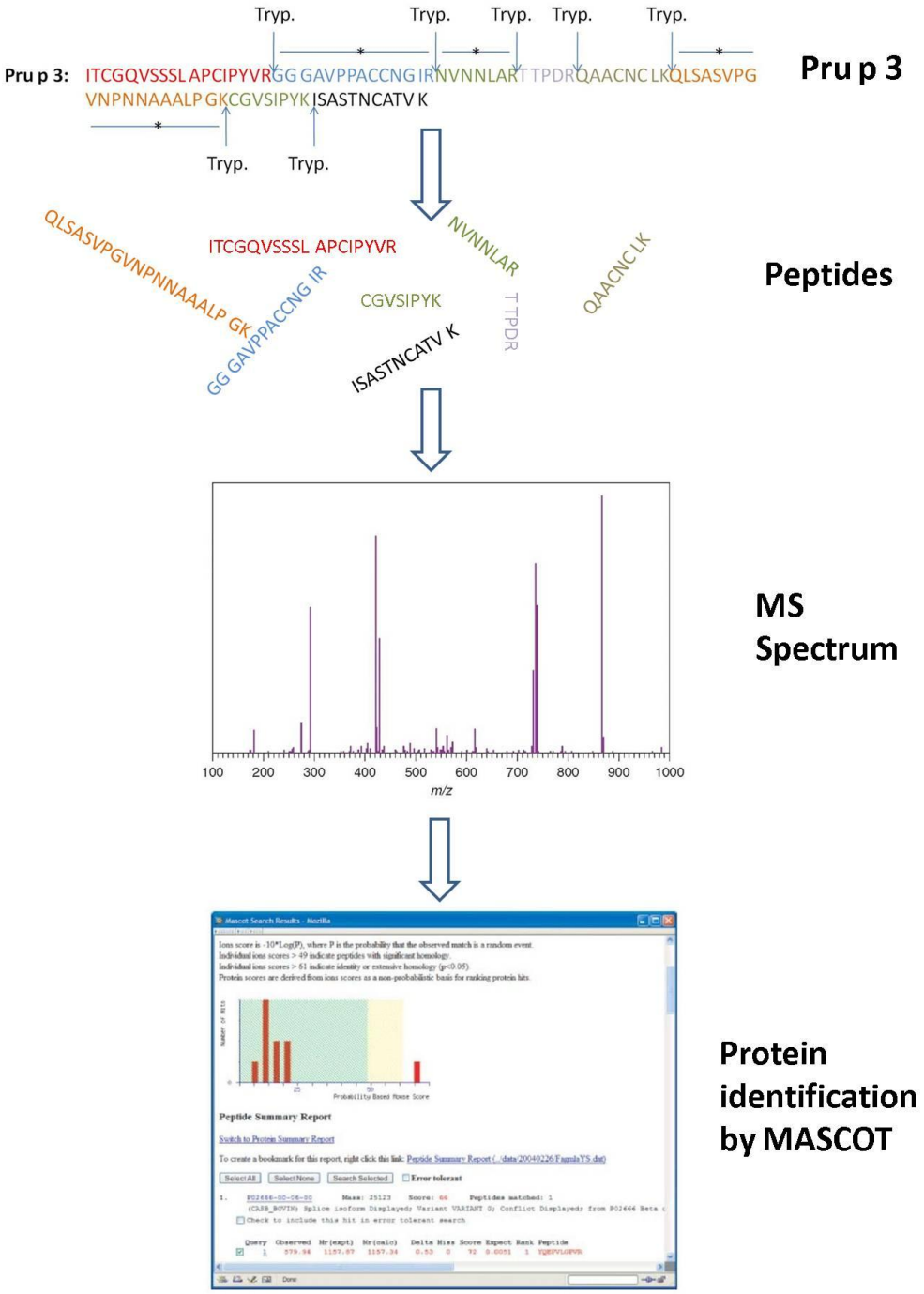


Fig.2.7: Schematic representation of mascot database search for candidate protein.

2.4 Immunochemical methods

2.4.1 Materials

All the chemicals used were of analytical grade. Nunc Maxisorp 96 well microtitre plates were obtained from Thermo Scientific, Loughborough, Leicestershire, UK. Disodium hydrogen phosphate, sodium chloride, potassium dihydrogen phosphate, potassium chloride, Tween 20,

alkaline phosphatase conjugated anti – rabbit IgG raised in goat, horse radish peroxidise conjugated anti – rabbit IgG raised in goat, Sigma FAST BCIP/NBT (5 – bromo – 4 – chloro – 3 – indoyl phosphate/ nitro blue tetrazolium tablets), orthophenylene di-amine di-hydrochloride (OPD), 3,3',5,5', tetramethylbenzidine (TMB) were obtained from Sigma, Dorset, UK. Skimmed milk powder (Sainsbury's, Norwich, UK) was obtained from a local supermarket.

2.4.2 Enzyme linked immunosorbent assay (ELISA)

2.4.2.1 Principle of ELISA

Enzyme linked immunosorbent assay (ELISA) is an analytical technique designed to determine antigens and antibodies within biological samples qualitatively and quantitatively and was developed by Engvall and Perlman nearly four decades ago (Engvall and Perlmann, 1971). One of the reactants is immobilized covalently or through adsorption on a solid support such as polyvinyl chloride or polystyrene as monomolecular surface layer in such a way that it does not alter the biological activity of the molecule. This provides advantage in removing bound from unbound antibody or analyte. The addition of partner reactant binds them to immobilized reactant and unbound reactant is removed by washing. The extent of reaction is measured by adding enzyme conjugated antibodies or target analyte which convert a colourless substrate into measureable (usually coloured) product which is proportionate to the antigen – antibody reaction (Perlmann and Perlmann, 2001, Paulie et al., 2005).

There are various ELISA formats. For example, an "Indirect ELISA" is used to determine the presence of specific antibodies using immobilized (adsorbed) analyte. The unbound antibodies are removed by washing and the bound antibodies are detected by enzyme conjugated anti-immunoglobulins, the amount of antibody being directly proportional to the amount of substrate converted to measureable product.

The method employed in this study was "Direct Competition ELISA" where the analyte is first immobilized to the solid plate such as 96 well microtitre plate. Serial dilutions of known quantities of analyte along with unknown sample of antigen are incubated with capture antibody and then transferred to the analyte coated plate. Depending upon the proportion of capture antibody to the known analyte, free and immobilized analytes compete for capture antibody into the reaction mixture. The unbound mixture on capture antibody and analyte is then removed by washing followed by adding detection antibody usually conjugated with enzyme such as horse reddish peroxidise. The free detection antibody is removed by washing and the quantity of this bound

antibody is determined by adding a suitable substrate such as 3,3',5,5' tetramethylbenzidine (TMB) which develops a reaction product blue in colour and turns into yellow when the reaction is topped by sulfuric acid. The coloured reaction product is measured spectrophotometrically in a plate reader at 450 nm which is inversely proportion to the quantity of unknown analyte within the sample (Perlmann and Perlmann, 2001, Paulie et al., 2005). A schematic representation of the assay is given in the Fig. 2.8.

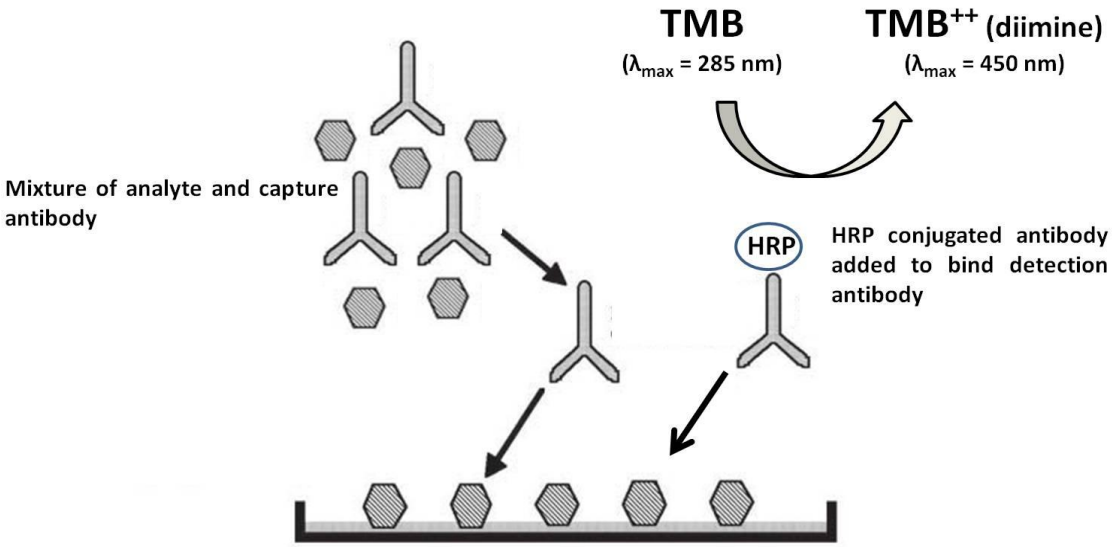


Fig.2.8: indirect competition ELISA for determination of analyte.

2.4.2.2 Method

Indirect competition ELISA was used in this study to measure the levels of Pru p 3 in samples. In a preliminary experiment, ELISA was established to optimize the concentrations of various parameters such as coating concentration, concentration of primary and secondary antibody and suitable buffer to coat the protein (details of the method explained in chapter 4). Phosphate buffer saline (PBS: 9.5 mM phosphate 137 mM NaCl) filtered through 0.22 µM filter disc was used to coat the plates. The Nunc maxisorb 96 well microtitre plates (Thermo Scientific, Loughborough, Leicestershire, UK) were coated overnight with 200 µL of 0.5 µg/ mL Pru p 3 in PBS. The next day, plates were washed three times with PBST (0.1% Tween 20 in PBS). The coated plates were tapped to remove the liquid and air dried before wrapping into cling film. The plates were stored at -80°C until required.

For ELISA, the coated plates were kept at ambient temperature to warm up and washed three times with PBST. The standard dilution of Pru p 3 covering 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 and 0.000001 µg/ mL in PST were prepared fresh just before use. Primary antibody (capture

antibody: rabbit IgG raised against Pru p 3) was prepared in PBS containing 0.1% (v: v) tween (PBST) in 1:500,000 dilution. A volume of 100 µL of this antibody solution was mixed with 100 µL of sample or standard and applied to the 96 well plate previously coated with Pru p 3. The plates were incubated overnight at 4°C followed by washing three times with PBST. The plates were tapped to remove excessive fluid and air dried. A volume of 200 µL of horse radish peroxidise conjugated anti – rabbit IgG (detection antibody: 1:5000 (v: v) in PBST) was applied to each well in the plate and incubated at room temperature for three hours. The plates were then washed three times with PBST, tapped and air dried. O – Phenylene diamine dihydrochloride (OPD) dissolved at 0.4 mg/ mL in 50 mM Phosphate citrate buffer containing 0.03% sodium perborate (Sigma, Dorset, UK) or 3,3',5,5'-tetramethyl benzidine (TMB, used directly) was applied in each well in 200 µL volume. The plates were kept in dark for 30 minutes and the reaction was stopped by adding 50 µL of 3 M HCl/ H_2SO_4. Finally, the plates were read at 450 nm in a microtitre plate reader.

2.4.3 Immunoblotting

2.4.3.1 Principle

Blotting is a method of transfer of a target molecule onto suitable solid supports such as membranes to immobilize them and was first developed for proteins by (Towbin et al., 1979). It allows analysis of macromolecules such as proteins from complex mixtures after separation by electrophoresis (Kurien and Scofield, 2003, Page and Thorpe, 2002, Gravel, 2002). In case of protein blotting (western blotting), the most widely used method is the separation of proteins by means of SDS-PAGE or isoelectric focussing, followed by electrophoretic transfer onto an immobilizing membrane such as polyvinylidine fluoride (PVDF) or nitrocellulose. When combined with immunodetection after transfer to a membrane, it allows protein identification even of low abundance polypeptides. The efficiency of the blot depends upon the highly efficient elution of the protein from the gel and binding efficiency to the membrane. High molecular weight proteins may transfer poorly and can require either disruption of the gel or partial proteolysis prior to transfer (Kurien and Scofield, 2003). Various methods have been employed to transfer proteins onto membranes such as simple diffusion where the membranes is sandwiched with gel and incubated for a couple of hours; vacuum blotting where a suction power is applied or most commonly used electroblotting (Kurien and Scofield, 2003). Electroblotting is carried out either by wet blotting by immersing the gel sandwich into transfer buffer or by semidry blotting where the sandwich is placed between two supports equilibrated previously in transfer buffer and the sandwich placed between electrodes (Kurien and Scofield, 2003). Dot immunobinding assay introduced by (Sumi et

al., 1999) is also a rapid method of protein detection with the help of antibodies. In this technique, protein is placed on membranes directly followed by detection with antibody. The sensitivity of the assay is 100 ng/ mL (Kurien and Scofield, 2003).

2.4.3.2 Methods

Proteins were resolved on SDS-PAGE gels as previously described (section 2.1.2). Two thick pads (BioRad, Hertfordshire, UK) and nitrocellulose membrane (Biorad, Hertfordshire, UK) previously equilibrated in transfer buffer (39 mM Glycine, 48 mM Tris base, 0.0375% (w: v) SDS and 20% (v: v) methanol in water) along with the SDS-PAGE gel for 15 minutes. The gel sandwich was prepared by placing the membrane over one thick pad followed by placing the gel onto the membrane. Finally, the second thick pad was placed on top of the gel. During the sandwich making, it was made sure that no air bubble is trapped between the layers. This was assured by rolling each layer with the help of a glass rod. The gel sandwich structure is given in Fig. 2.9.

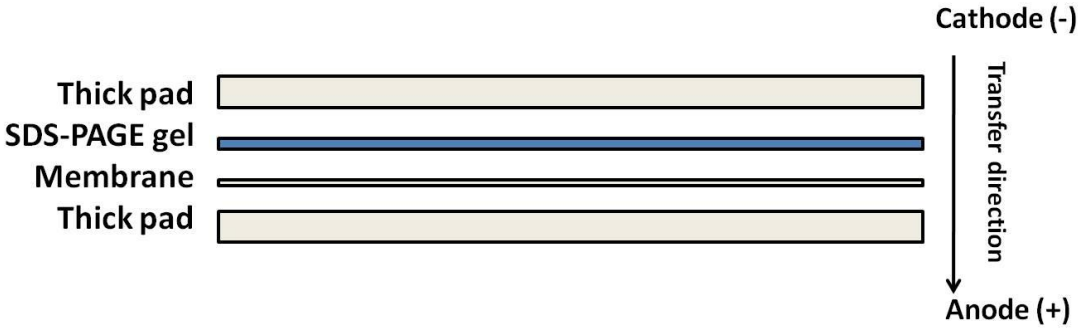


Fig.2.9: Gel sandwich prepared with two thick pads with gel and membrane sandwiched between them previously equilibrated in transfer buffer.

The sandwich was placed in a semidry electroblot unit (BioRad, Hertfordshire, UK) and blotting was carried out at 15 V for 35 minutes. At the end of the run, the components of the sandwich were separated and the membrane removed. The membrane was blocked by immersing in 5% (w:v) skimmed milk powder in PBST and kept with shaking for 1 h at room temperature. After blocking with milk powder, the membrane was washed three times with PBST, each time for 5 minutes. Primary antibody (rabbit polyclonal IgG raised against Pru p 3 were kind gifts from Philippe Delahaut, CER Groupe, Département Santé, Marloie, Belgium) diluted in PBST at 1:10,000 (v:v) was applied to the blot and kept shaking at room temperature for 1 hour. At the end of primary antibody reaction, the blotting membrane was washed three times with PBST as described above.

Secondary antibody (Alkaline phosphatase conjugated anti – rabbit IgG) was diluted in PBST at 1:1000 (v:v) and applied to the membrane for one hour with shaking at room temperature. At the end of secondary antibody reaction, the excess antibody was removed by washing three times with PBST. BCIP/NBT was prepared by dissolving 1 substrate tablet in 10 mL water and dissolved in dark. The substrate (BCIP/NBT) solution was applied to the membrane for 2-3 minutes or until the bands of Pru p 3 appeared.

2.5 Data handling

2.5.1 Data analysis for ligand binding

The data was analyzed by using GraphPad Prism v.5.01. Standard deviation were calculated and shown in the graps as error bars.The fluorescence intensity was plotted against total concentration of CPA or displacing ligand. The curve was fit with hyperbolic one site binding function. For displacement assay, the curve was fit by competitive binding function for one site taking into account for $LogIC_{50}$. The curve fitting returned K_a values for CPA which are the binding constants for LTPs and IC_{50} values for displacing ligands. Cheng-Prusoff equation was used to calculate K_i for displacing ligands. The binding energy was calculated according to Gibb's free energy calculated as:

Where ΔG = Gibb's free energy

R = Universal gas constant
T = Absolute temperature
K_d = Dissociation constant

2.5.2 Data analysis for CD

The data was analyzed by CDPro using two different programs namely CDSSTR and ContinLL. Each program utilizes seven different sets of reference namely IBasis 1 to IBasis 7.

3 Purification of lipid transfer proteins (LTPs)

3.1 Introduction

LTPs are found in the extracellular matrix (Meijer et al., 1993) of peel and pulp of fruits belonging to the Rosaceae family and in the bran of cereal grains such as wheat (Borges et al., 2006). Many LTPs are allergens (Zuidmeer and Ree, 2007), some examples of them are Pru p 3 (peach), Pru du 8 (almond), Pyr c 3 (pear), Fra a 3 (strawberry), Rub i 3 (raspberry), Pru av 3 (cherry), Mal d 3 (apple), Pru ar 3 (apricot) and Pru d 3 (plum) (Fernández-Rivas, 2009, Borges et al., 2006, Scheurer et al., 2004). In most cases, the skin of fruit, such as peach and apple contains higher concentrations of LTPs than does pulp, where as in other cases, such as in apricot and plum, they are equally distributed in peel and pulp (Borges et al., 2006). The amount of LTP in peach is 8.4 µg/ g of peel and 3.4 µg/ g in pulp (Borges et al., 2006). The level of expression varies greatly depending upon the variety, such as in the case of apple where LTP ranges from 2 – 17.3 µg/ g of peel and 0.5 – 1.8 µg/ g of pulp (Borges et al., 2006).

Confocal laser scanning microscopy after immunocytochemical staining with antibodies raised against Pru p 3 showed that this protein is highly abundant in the thick walls of the downy hairs covering the epidermal cell layer (Borges et al., 2006). The cytoplasm of the epidermal cells is also rich in this LTP where the highest abundance is found at the inner part of the cell wall.

LTPs have been purified from the soluble extracts of various plant tissues by using a combination of gel filtration, cation exchange chromatography and reverse phase HPLC (Kader, 1996). The most widely used method for purification of LTP from fruits was described by Bjorksten and co-workers in 1980 where the fruit extract is made in phosphate buffer containing PVPP, sodium diethyl dithiocarbamate and sodium azide (Björkstén et al., 1980). The LTP (in this case Pru p 3 from peach) was concentrated from such extracts by means of ammonium sulphate precipitation followed by chromatographic separation (Pastorello and Trambaioli, 2001). Recently published protocols (Gaier et al., 2008, Carnés et al., 2002, Pravettoni et al., 2009, Pastorello et al., 2001, Oberhuber et al., 2008) use peel as a starting material instead of pulp giving higher initial LTP content with less interfering components such as sugars and lipidic components that may affect chromato-graphic resolution. After freezing in liquid nitrogen to prevent undesirable modification of the protein by e.g. proteases released by tissue disruption, the tissue is ground whilst frozen into extraction buffer. The resulting extract is subjected to 95% ammonium sulphate precipitation and the precipitate collected and dialysed to remove excess salt. The resulting enriched extract is then applied to a

cation exchange column and eluted with 20 mM MES pH 5.6. The partially purified Pru p 3 is then subjected to a round of gel filtration chromatography (Gaier et al., 2008). A defatting step was used in some cases where the ground skin was defatted first with acetone at 4°C followed by 1:3 (v:v) volume of ethanol: ether (Diaz-Perales et al., 2000).

In contrast, extraction of wheat LTP did not require as extensive precautionary steps to prevent modification by polyphenols. Two different isoforms of LTPs have been purified from barley, one native LTP while the other being an adduct of lipid (Lindorff-Larsen et al., 2001). The bran or wheat flour was defatted with either cold acetone (Palacin et al., 2007) or hexane to remove the adduct with LTP and an extract made in water and clarified by centrifugation. The extract was then brought to 20 mM MES pH 5.6 and fractionated by cation exchange chromatography using a cation exchange resin followed by gel filtration with Sephadex G50. LTP enriched fractions were further purified to homogeneity with reverse phase HPLC on a 5-µm/300˚A bonded silica. The yield of LTP thus obtained was 250 µg/ g of bran (Charvolin et al., 1999, Tassin-Moindrot et al., 2000). Palacin and co-workers (Palacin et al., 2007) used a modified method to purify wheat LTP by making an extract in 100 mM Tris-HCl and 10 mM EDTA pH 7.5. This was then fractionated by cation exchange chromatography followed by reverse phase HPLC. The purified protein was analysed by SDS-PAGE and immunodetection.

This section explains the methods used to purify Pru p 3 and Tri a 14 in their native forms and their characterization by circular dichroism spectroscopy and mass spectrometry required in this study.

3.2 Chromatographic methods

3.2.1 Materials

LTP was purified from peach (Pru p 3) and wheat (Tri a 14). Fresh mature peaches and wheat bran (Nature's Harvest) were purchased from a local market in Norwich, UK. Sodium dihydrogen orthophosphate, sodium chloride, ammonium sulphate, MES were purchased from Sigma, UK and were of analytical grade.

Throughout the experiments, 18.2 MΩ ultra pure water was used which was obtained by reverse osmosis followed by purification with Barnsted Nanopure (Thermo Scientific, Loughborough, UK). The purifier works in four steps, ion exchange, membrane deionization, filtration and oxidation. Ion exchange mainly demineralises the water along with scavenging any organic material. Membrane demineralization works on the principle of reverse osmosis and further demineralises the water.

Finally, ultra filtration using carbon filters removes any further material before oxidation/ disinfection by UV light to prevent microbial growth.

3.2.2 Purification of peach LTP (Pru p 3)

The purification of Pru p 3 was done by using a combination of cation exchange chromatography and gel filtration. The purification steps are given in the form of a scheme in Fig. 3.1.

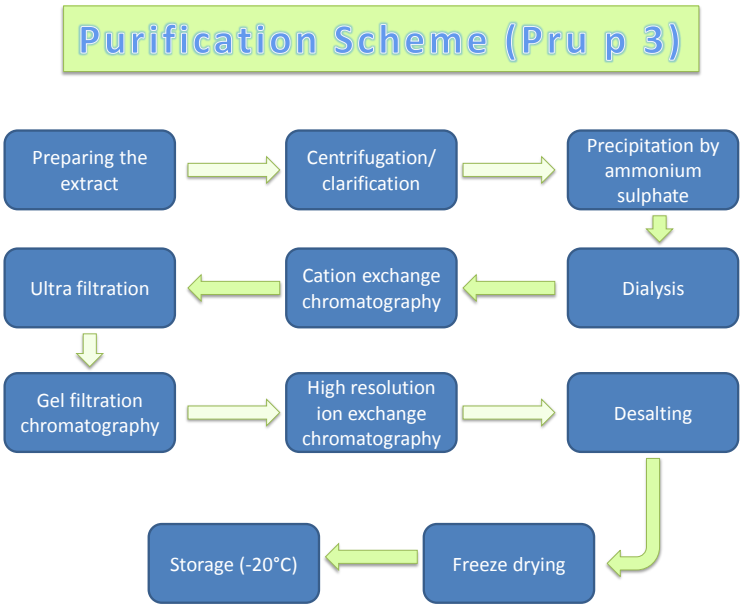


Fig. 3.1: Purification scheme for Pru p 3

3.2.2.1 Extract preparation

LTP was isolated from peach peel as it contains approximately double the protein content and seven times higher content of target protein than pulp (Carnés et al., 2002). Therefore, peel was removed from the pulp by using a domestic knife such that the thickness was kept to 2-3 mm. Immediately after removal, peel was frozen by immersion in liquid nitrogen and stored at -80˚C until required.

An extraction buffer comprising NaH_2PO_4 (50 mM) pH 7.0 containing 20 mM sodium diethyl-dithiocarbamate, 2 mM EDTA and 0.02% (w:v) sodium azide, was prepared fresh immediately before use. PVPP was added to a concentration of 3% (w:v) to extraction buffer as a suspension at least 10 minutes prior to addition of peel to allow it to hydrate and equilibrate. Frozen peel was ground in liquid nitrogen in a Waring blender (Waring Commercial, Connecticut, USA) to a fine powder. The powder was allowed to warm at ambient temperature to -10˚C before adding extraction buffer containing PVPP at a ratio of 2:1 (w: v) to the peel and homogenizing in the

Waring blender for two minutes to rupture tissue. Finally, the suspension was stirred at ambient temperature with a paddled top stirrer for one hour.

After extraction, the homogenate was centrifuged at 1700 x g for 30 minutes at 10˚C, the supernatant collected and the pellet discarded. Ammonium sulphate was added to the supernatant to 40% saturation by gradual addition of solid ammonium sulphate at 10˚C with subsequent stirring for 30 minutes. The suspension was centrifuged again at 1700 x g for 30 minutes at 10˚C, the supernatant retained and the pellet discarded. LTP was then precipitated from the 40% ammonium sulphate supernatant by gradual addition of solid ammonium sulphate to 95% saturation at 10˚C and subsequent stirring for 30 minutes. Finally, the suspension was centrifuged at 1700 x g for 45 minutes and the pellet retained after decanting the supernatant.

The pellet was re-suspended in deionised water and dialyzed against 2L of water using a 3.5 KDa MW cut off dialysis membrane (Spectra/Por, Fisher Scientific, Loughborough, UK) at room temperature. The dialysis was carried out for 3.5 hours with a change of dialysing water after each hour. Finally, the dialysate was collected and brought to 20 mM MES by the addition of solid MES. The pH was adjusted at 6.0 using 5 M NaOH and the extract was centrifuged at 22100 x g for 30 minutes at 10˚C to obtain a clear supernatant.

3.2.2.2 Ion exchange chromatography

Chromatography was performed using BioCad Sprint Perfusion Chromatography system (Applied Biosystems, Farmingham, USA). All solutions were filtered through 0.22 μm filter (Millipore, Watford, UK) using a vacuum filtration assembly and kept refrigerated until use. All the buffers were stored no more than one week.

The running buffer (A) was prepared by dissolving MES in deionised water at a concentration of 100 mM and the pH adjusted to 6.0 with 5 M NaOH. The running buffer (B) was prepared by dissolving NaCl at a concentration of 3 M in deionised water. Pure HPLC grade water was used as running buffer (C).

A column (13.5 x 2.5 cm, 66.7 mL volume) was packed with SP-Sepharose (Amersham Biosciences, Uppsala, Sweden) and equilibrated in 20 mM MES pH 6.0 (20% (v: v) buffer A and 80% buffer C (v:v)) at a flow rate of 5 mL/ min until a stable baseline of UV absorbance and conductivity was obtained. A volume of 20 mL of the extract was applied to the column, and the column was washed with equilibration buffer up to 0.5 column volume (CV). A linear gradient

from 0 – 0.5 M NaCl (20% (v:v) buffer A, 0 - 17% (v:v) buffer B in water (buffer C)) was applied over 2.5 column volumes followed by a step gradient of 1 M NaCl over 0.5 column volume to elute more strongly bound proteins. Eluent was monitored for protein by UV absorbance at 280 and 220 nm, and collected in 5 mL fractions and analyzed by SDS-PAGE to identify the molecular weight of the proteins present in various protein-containing peak fractions. Fractions containing approximately Mr 9 kDa proteins with low absorbance at 280 nm as compared to 220 nm were pooled and stored refrigerated until required for further purification of LTP. Fractions were stored no more than three days.

The pooled fractions from ion exchange chromatography containing 9 kDa protein as identified by SDS-PAGE containing putative Pru p 3 were filtered through 0.45 μm filter disc and the filtrate was concentrated to 10 fold between 0.7 – 1.0 atm pressure at ambient temperature by ultrafiltration through a 1 kDa cut off membrane in an Amicon ultra filtration assembly (Millipore, Watford, UK).

3.2.2.3 Gel filtration

The gel filtration buffer was prepared by dissolving ammonium acetate in deionised water at a concentration of 100 mM. Sodium azide was added at a concentration of 2 mM to prevent microbial growth. No pH adjustment was required for this solution, although the pH was monitored to check it was at 6.7. The solution was filtered through 0.22 μm filter disc and refrigerated until required. The buffer was stored for no more than one day.

Standard pre-packed preparative grade column Superdex 75 (1.6 cm x 60 cm, 120.637 mL bed volume, Pharmacia, Uppsala, Sweden) was used to fractionate proteins by molecular weight from the mixture of proteins obtained by cation exchange chromatography. The column was equilibrated in gel filtration buffer at a flow rate of 1 mL/ min prior to sample loading until baseline stability of UV absorbance and conductivity. Samples were applied in 2 mL volumes and fractionated under the same buffer and flow rate. Eluent was collected in 2 mL fractions and monitored for protein by UV absorbance at 280 and 220 nm and SDS-PAGE. The pooled fractions were freeze-dried and stored frozen at -20°C until required.

3.2.2.4 High resolution ion exchange chromatography

The running buffer A was 25 mM MES, pH 6.5 while running buffer B was 1 M NaCl. All the solutions were filtered through 0.45 μm filter disc and stored refrigerated conditions until required. All the solutions were stored no more than one week.

Samples obtained from gel filtration chromatography were a mixture of a Mr 9 kDa protein (putative Pru p 3) along with a minor contaminant of Mr 7 kDa just below the LTP (Fig. 3.5). This contaminant was removed by high resolution ion exchange chromatography. A preparative column of Poros HS – 20 (0.46 cm x 10 cm, 1.662 mL bed volume, Applied Biosystems, Farmingham, USA) was packed and equilibrated in 25 mM MES buffer (buffer A) at pH 6.5 at a flow rate of 4 mL/ min. The freeze dried material obtained from gel filtration was suspended in water, adjusted at 25 mM MES pH 6.5 and applied to column followed by washing up to 2 CVs with buffer A. A linear gradient of 0 – 0.3 M NaCl (0 – 30% (v:v) buffer B, 100 – 70% (v:v) buffer A) was applied over 15 CV followed by a step gradient of 0.3 M NaCl to 1 column volume to elute strongly bound proteins. Fractions containing putative Pru p 3 were identified by SDS-PAGE and pooled.

3.2.2.5 Desalting

The purified protein was in a solution of buffer and salt which were removed by desalting. A preparative column of Sephadex G15 (Sigma, St. Louis, USA) 13.5 cm x 2.5 cm (column volume 66.7 mL) was equilibrated in deionised water at a flow rate of 2 mL/ min until a stable baseline regarding UV absorbance and conductivity was obtained. A volume of 5 mL of the protein solution was loaded to the column and eluted with deionised water. Fractions were collected in 3 mL volumes and monitored for protein by UV absorbance (Fig. 3.8). The fractions with high UV absorbance corresponding to a single peak were pooled and freeze dried.

3.2.3 Purification of wheat LTP (Tri a 14)

Purification of Tri a 14 was achieved by a combination of cation exchange chromatography and gel filtration. The purification scheme is given in Fig. 3.2.

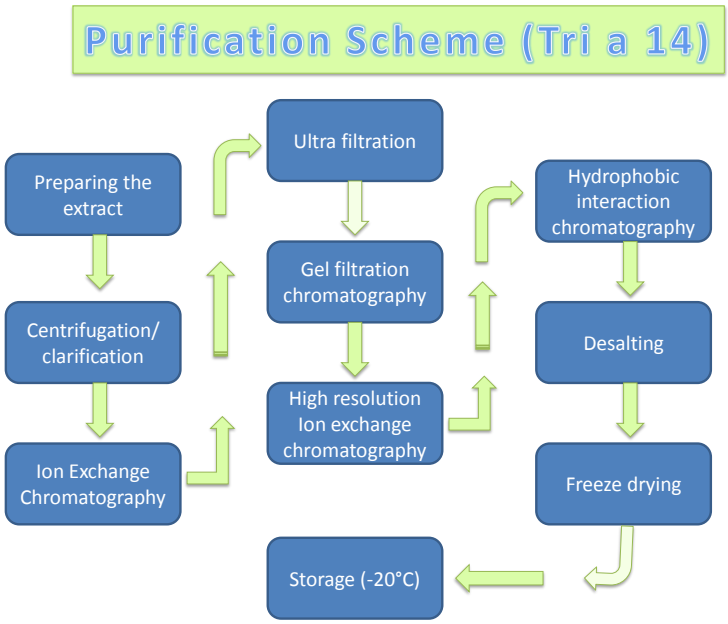


Fig. 3.2: Purification scheme for Tri a 14

3.2.3.1 Defatting the bran

Wheat bran was suspended in HPLC grade hexane at a ratio of 1:10 (w:v) in a stainless steel beaker and stirred in a fume hood at ambient temperature for 1 h. The extract was filtered through a glass microfiber filter disc in a Buchner funnel connected to a flask under vacuum. The bran was collected from the funnel and re-suspended in hexane at the same ratio and stirred under the same conditions for the same length of time. After a second filtration, the bran was finally suspended again in hexane for final extraction with the same conditions and filtered. The defatted bran obtained in this way was kept under the fume hood overnight to remove residual hexane. The next day, bran was sealed in a plastic bag and kept stored at ambient temperature in the dark.

3.2.3.2 Extract preparation

Defatted bran (100 g) was suspended in 1 L of deionised water containing 33 g of PVPP previously soaked and suspended in water at least 10 minutes prior to extraction to equilibrate. The whole suspension was stirred at ambient temperature with a paddle stirrer for one hour. After extraction, the suspension was filtered, squeezed through a Miracloth (Merck Biosciences, UK) and clarified

by centrifugation at 1700 x g at 10˚C for 30 minutes. The supernatant was retained and brought to 20 mM MES pH 6.0 by addition of solid MES and filtered through glass microfiber filter disc (Whatman International Ltd Maidstone, England) in a Buchner funnel connected to a vacuumed flask. The extract was freshly prepared each time prior to further purification.

3.2.3.3 Ion exchange chromatography

The running buffer (A) was 100 mM MES pH 6.0 while running buffer (B) was 3 M NaCl. All solutions were filtered through 0.22 μm filter disc in a vacuum filtration assembly and kept under refrigerated at 4˚C until use. A preparative cation exchange column (2.5 cm x 13.5 cm, 76 mL bed volume) was packed with cation exchange resin (SP-Streamline, Pharmacia, Uppsala, Sweden). The extract prepared was applied to the column previously equilibrated with 20 mM MES pH 6.0 at a flow rate of 10 mL/ min. The column was washed with the same equilibration buffer to 0.5 CV. A step gradient of 1 M NaCl was applied over 2.5 CV to elute the bound proteins. The eluate was monitored for protein content by UV absorbance at 220 nm and by SDS-PAGE. The elution yielded a single peak over a few fractions which were pooled and kept frozen at -20˚C until further processing.

The pooled Streamline column fractions were filtered through 0.45 μm filter disc and concentrated by ultra filtration through 1 kDa membrane filter in an Amicon ultra filtration assembly (Millipore, Watford, UK).

3.2.3.4 Gel filtration

The gel filtration buffer was 100 mM ammonium acetate, 0.02 % (w:v) sodium azide in deionised water. No pH adjustment of this solution was required although the pH was monitored to ensure it was 6.7. The solution was filtered through 0.22 μm filter disc and refrigerated at 4˚C until required.

The pooled fractions obtained from ion exchange chromatography were further purified by gel filtration chromatography using a Superdex 75 column (1.6 cm x 60 cm, 120.6 mL bed volume, Pharmacia, Uppsala, Sweden). The column was equilibrated in gel filtration buffer at a flow rate of 1.2 mL/ min and the sample applied in 2.5 mL injections. Fractionation was achieved in the same buffer at 1.2 mL/ min and the eluate monitored for protein by UV absorbance at 220 nm and SDS-PAGE and those fractions containing 9 kDa protein (putative LTP) were pooled. Finally, the pooled fractions were freeze dried directly due to the volatile nature of the buffer. The freeze dried material was stored at -20°C until required.

3.2.3.5 High resolution ion exchange chromatography

All solutions were filtered through 0.22 μm filter disc in a vacuum filtration assembly and refrigerated at 4˚C until required. All the buffers were stored no more than one week.

The running buffer (A) was prepared by dissolving MES in deionised water at a concentration of 100 mM. The pH was adjusted at 5.5 with 5 M NaOH. The running buffer (B) was prepared by dissolving NaCl at a concentration of 3 M in deionised water. A POROS HS-20 column (Applied Biosystems, Farmingham, USA) was used with the same parameters as described for the purification of Pru p 3. The column was equilibrated in 25 mM MES pH 5.5 (25% (v/v) buffer A in water) while the sample was adjusted to contain 25 mM MES pH 5.5 by addition of solid MES. Freeze dried sample obtained after gel filtration was dissolved in running buffer at a concentration of 4 mg/ mL and applied to the column in 2 mL volumes at a flow rate of 4 mL per minute followed by washing up to 10 column volumes. Bound proteins were eluted by applying a gradient from 0 – 0.25 M NaCl (25% (v/v) buffer A, 0 – 8.3% (v:v) buffer B in water) over 20 column volumes. The eluate was monitored for presence of Tri a 14 by absorbance at 220 nm and SDS-PAGE.

3.2.3.6 Hydrophobic interaction chromatography

Fractions obtained from high resolution ion exchange chromatography (partially purified Tri a 14) contained minor contamination from a Mr 7 kDa polypeptide (possibly Mr 7 kDa LTP of wheat) which was removed by hydrophobic interaction chromatography.

All the buffers were filtered through 0.22 μm filter disc in a vacuum filtration assembly and kept refrigerated at 4°C until required. Running buffer A was 100 mM Tris pH 7.4 while buffer B was 3.5 M ammonium sulphate. A preparative column of HP 2 resin (Applied Biosystems, Farmingham, USA) was packed as 0.46 cm x 10 cm, 1.66 mL bed volume. The column was equilibrated in 20 mM Tris, 2.8 M ammonium sulphate (20% (v:v) buffer A, 80% (v: v) buffer B) until a stable baseline of UV absorbance at 220 nm of the eluate was obtained. The sample obtained from high resolution ion exchange chromatography containing putative Tri a 14 was brought to 3 M ammonium sulphate by addition of solid ammonium sulphate and applied to the column at a flow rate of 4 mL/ min. The column was then washed with 3 CV of equilibration buffer and the putative Tri a 14 eluted using a linear decreasing gradient of 2.8 – 0 M ammonium sulphate over 20 CV followed by a step gradient of 0 M ammonium sulphate over 3 CV. Fractions were analyzed by SDS-PAGE and those not contaminated by the Mr 7 kDa protein were pooled and kept frozen as pure wheat lipid transfer protein, Tri a 14.

3.2.3.7 Desalting

The putative Tri a 14 preparation obtained was in solution containing a high concentration of ammonium sulphate, which would dehydrate a normal desalting bed of Sephadex G15. To remove partial salt, the protein solution was brought to 3 M ammonium sulphate by dissolving solid ammonium sulphate into the protein solution and loaded on Phenyl-Sepharose column (13.5 x 2.5 cm, 66.7 mL, Amersham Biosciences, Uppsala, Sweden) previously equilibrated in 20 mM Tris 3 M ammonium sulphate pH 7.5. The bound protein was eluted with water applied as step gradient and then desalted with Sephadex G15 column as described in section 3.2.2.5. The desalted protein solution was freeze-dried and stored frozen at -20°C.

3.3 Characterization

Protein quantification was carried out by using BCA assay as described in section 2.1.5. The protein secondary structure was determined by Far UV – circular dichroism spectroscopy as described in section 2.1.6 while protein molecular mass and identification of primary structure was carried out by MALDI – ToF – MS as described in section 2.3.

3.4 Results

3.4.1 Purification and characterization of Pru p 3

Peach extract was made at low temperature to avoid reactions between Pru p 3 and other modifying components such as metal ions, phenolics and organic matter. The higher content of Pru p 3 was obtained by using peels instead of pulp along with the advantage of low sugar content. The homogenate containing sodium diethyl dithiocarbamate (DiECar) provided the advantages of prevention against reversible interaction between allergen and native phenolic compounds and irreversible interaction while these phenolics are oxidised (Björkstén et al., 1980). The use of PVPP provided protection from phenolic acids. The major advantage of using EDTA was to avoid metal interactions with LTP and inhibited metallo–proteases such as those produced by the microbial flora at the surface of peaches like *Xylella fastidiosa* (Fedattoa et al., 2006) while the use of NaN_3 prevented further growth of aerobic microbes during stirring.

3.4.1.1 Ion exchange chromatography

The pH of the extract (pH 6.0) was well below the isoelecric point (pI 8.89) which ensured highly charged state of the carboxyl groups. Pru p 3 is highly basic and was the last major peak (peak 3)

eluted in linear gradient range of elution. The last peak in the chromatogram represents the elution of non – protein compounds bound strongly to the column resin (Fig 3.3).

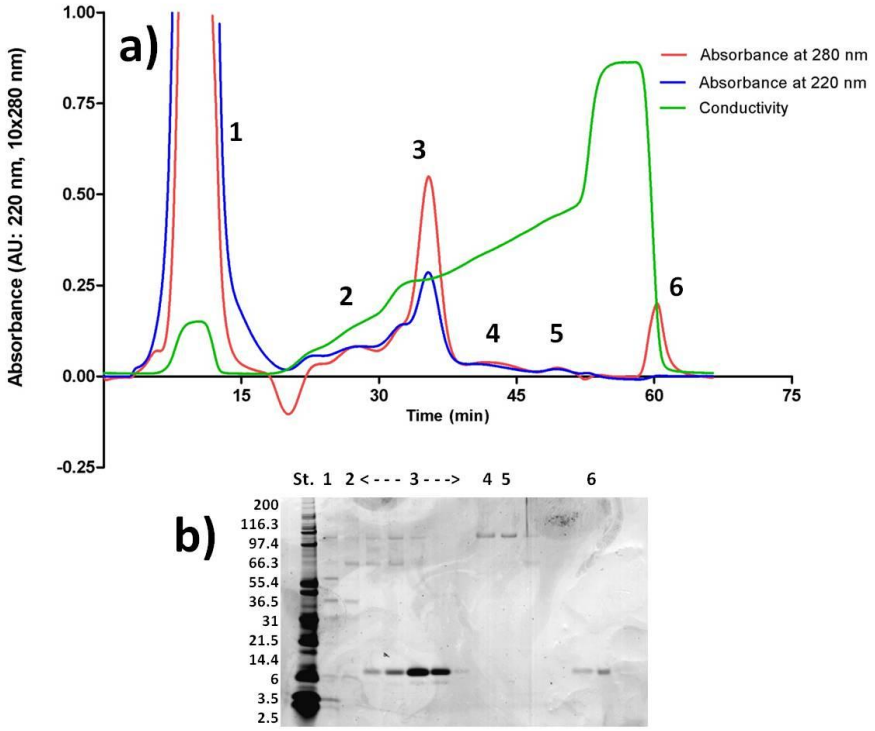


Fig.3.3: Ion exchange chromatography of putative Pru p 3. (a) Represents the chromatogram while (b) represents the SDS-PAGE of the fractions corresponding to various peaks in the chromatogram.

The first peak which is the largest peak of the chromatogram represents the proteins that were unbound to the resin under the flow conditions. Among four peaks fractionated within the linear region of gradient, the second peak (peak 3, Fig. 3.3) which was the major peak was identified as Pru p 3 according to the molecular weight as determined by SDS-PAGE. Finally, the organic matter and denatured proteins strongly bound to the resin came off the column at "step gradient" step representing the last peak of the column observed at 220 nm while absent in case of 280 nm.

3.4.1.2 Gel filtration

Superdex 75 gel filtration of the pooled fractions containing putative Pru p 3 was performed to remove major contaminants. The elution volume of the Pru p 3 was found to be 87 mL which is 0.60 CV of the bed after elution of its void volume (Fig. 3.4).

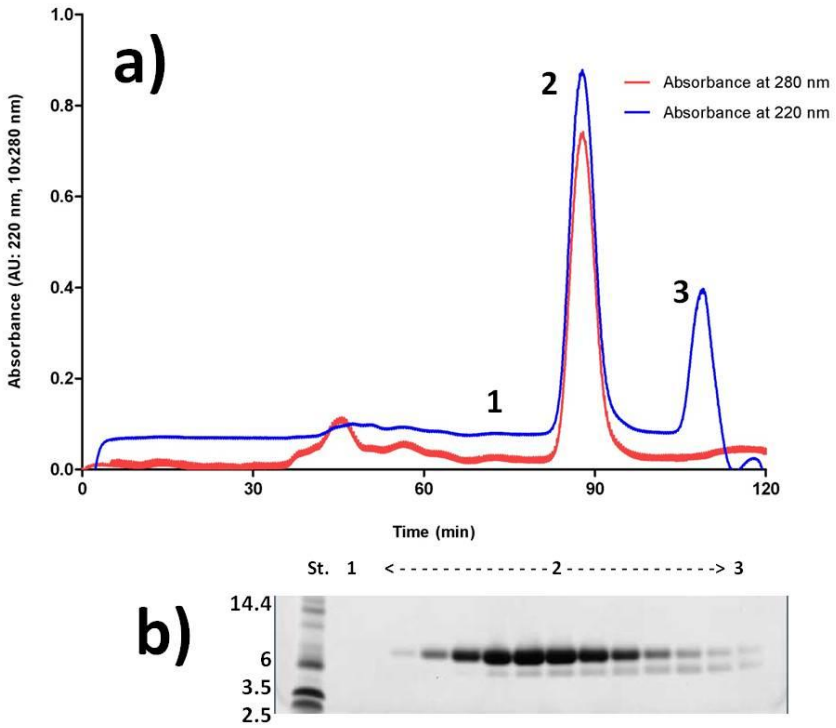


Fig. 3.4: Gel filtration of putative Pru p 3. (a) represents the chromatogram while (b) is the SDS-PAGE of the peaks marked on the chromatogram.

SDS-PAGE of the fractions containing putative Pru p 3 (peak 2, Fig. 3.4) showed two bands in the peak, a major one corresponding to Pru p 3 while a minor contaminant running faster but close to LTP. Both of these proteins were identified as Q9LED1, however the difference may be due to difference in the C-terminal residue which may be absent in the truncated protein appeared as a minor band (Cavatorta et al., 2009).

3.4.1.3 High resolution ion exchange chromatography

After gel filtration, the fractions contained a minor contaminant appearing smaller than Pru p 3 as estimated by SDS-PAGE. This contaminant was removed by high resolution ion exchange chromatography and a single SDS-PAGE band was obtained ensuring purity of the protein. The chromatogram with SDS-PAGE is given in Fig. 3.5.

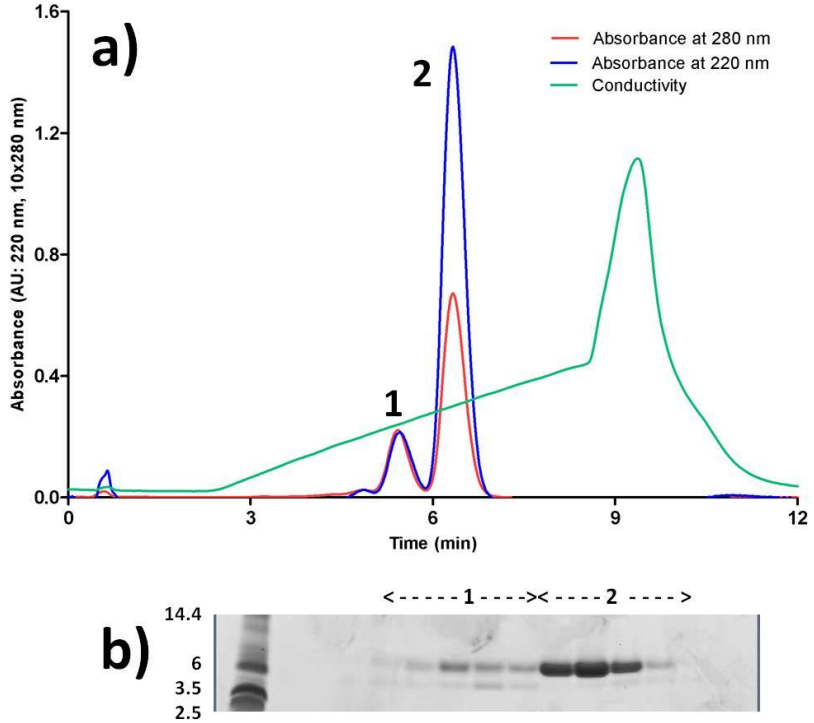


Fig. 3.5: High resolution ion exchange chromatography of putative Pru p 3. The chromatogram is given in (a) while the SDS-PAGE of the fractions in the chromatogram is given in (b).

The high resolution ion exchange chromatography successfully removed the contaminants as evident by SDS-PAGE with a single band. The fractions with high abundance of putative Pru p 3 and no contaminants (peak 2, Fig. 3.5) were pooled and desalted followed by freeze drying and storage at -20°C until required.

3.4.1.4 Far UV – CD

The CD spectrum of Pru p 3 has two negative peaks (minima) and a positive peak in the far UV region (Fig. 3.6). The two negative peaks at 208 nm and 222 nm and one positive peak at 192 nm are the characteristic peaks for an ordered α-helical structure. The CD spectrum of Pru p 3 is similar to that described for native folded Pru p 3 previously (Díaz-Perales et al., 2003).

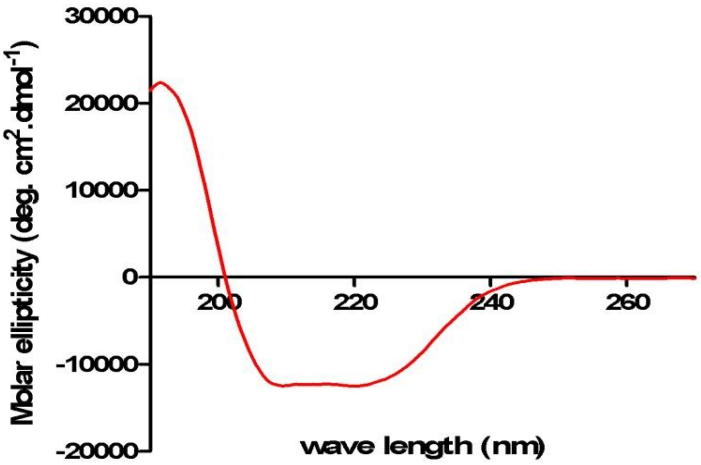


Fig. 3.6: CD spectrum of Pru p 3.

The data was analyzed by using CDPro and the proportion of helix and sheet along with turns and random coils were measured. Reference sets IBasis 3, 4, 6 and 7 were used by the program to calculate the structural components. On comparison, the secondary structure fractions returned by the software are presented in Table 3.1.

Table 3.1 Structural components of Pru p 3.

	CDSSTR					**ContinLL**			
	Helix	Sheet	Turn	Random		Helix	Sheet	Turn	Random
IBasis 3	0.41	0.15	0.18	0.21	**IBasis 3**	0.38	0.13	0.21	0.29
IBasis 4	0.39	0.16	0.18	0.27	**IBasis 4**	0.38	0.14	0.20	0.28
IBasis 6	0.40	0.13	0.16	0.30	**IBasis 6**	0.37	0.14	0.20	0.29
IBasis 7	0.40	0.14	0.15	0.31	**IBasis 7**	0.38	0.14	0.20	0.29

Analyzing by CDSSTR suggested the helical component of Pru p 3 to be 40% while the proportion of β – sheet was 15%. Random coil and turn comprise 28% and 17% respectively. ContinLL measured the helical structure as 38% while β – sheet as 14%. The random coil and turns remain at 28% and 20% respectively.

3.4.1.5 Mass spectrometry

Pru p 3 was characterized by MALDI – ToF – MS to determine the intact mass and purity. MALDI – ToF – MS of purified protein is given in Fig. 3.7. The spectrum contains predominantly a single sharp peak representing the purity of the sample. The intact mass was found to be 9136 Da.

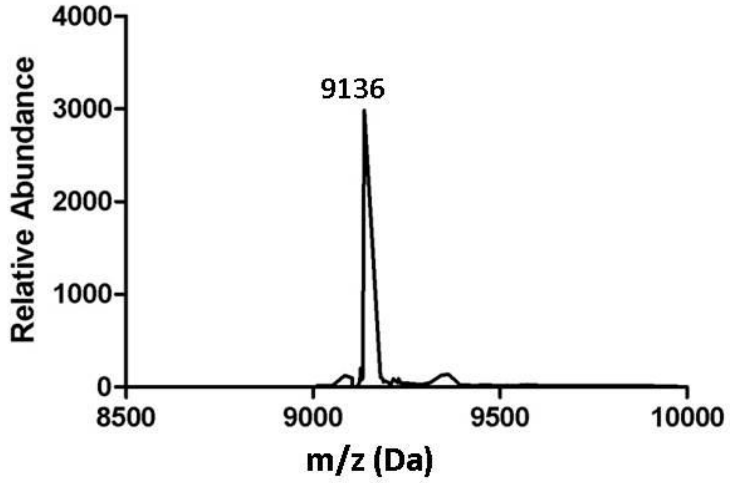


Fig. 3.7: MALDI – ToF-MS of Pru p 3.

In gel trypsin digestion of purified Pru p 3 followed by MALDI – ToF – MS returned 40 masses of the fragments (Table 3.2). The main fragments are represented in Fig. 3.8.

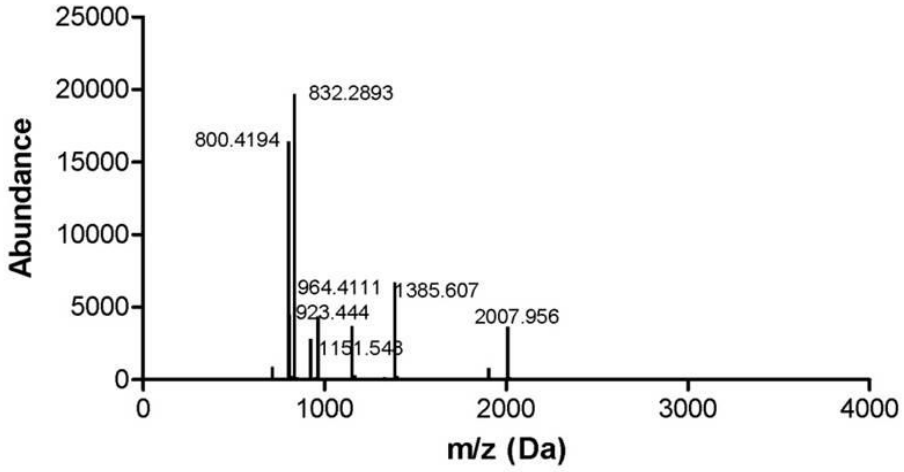


Fig. 3.8: MS spectrum representing peptide mass fingerprints of Pru p 3

Table 3.2: Peptide fragments obtained from tryptic digestion of Pru p 3

m/z	Intens.	m/z	Intens.	m/z	Intens.	m/z	Intens.
712.11	901.60	**846.27**	194.91	**964.41**	4401.86	**1401.61**	87.98
754.42	88.48	**847.27**	193.86	**975.42**	92.67	**1631.69**	78.73
800.42	16466.73	**862.26**	141.66	**978.43**	82.92	**1904.97**	815.86
804.26	4536.89	**864.27**	161.99	**1003.31**	87.67	**1929.06**	87.64
806.26	583.99	**866.43**	93.64	**1151.55**	3718.06	**1961.95**	50.05
818.26	270.04	**906.42**	117.22	**1165.56**	326.93	**1988.95**	49.14
820.25	242.80	**907.40**	159.98	**1328.59**	169.44	**2007.96**	3682.46
832.29	19695.11	**923.44**	2844.40	**1385.61**	6715.65	**2018.95**	184.65
834.29	2843.46	**947.38**	116.65	**1396.62**	67.18	**2021.96**	113.35
838.26	81.16	**949.25**	185.58	**1399.62**	251.62	**2023.94**	80.96

The fragments with Mr 800, 804, 832, 834, 923, 964, 1151, 1385 and 2007 Da were the major abundant peaks in the spectrum. The spectrum was submitted to Mascot database search engine for a probability-based MOWSE scoring algorithm which is used for peptide mass fingerprinting. The database calculated probability based ion scores of the known proteins by the specified enzyme and matched it with the experimental data. The MOWSE score for Pru p 3 was observed as 138 (>70, the lower significance level of probability based MOWSE score) which is highly significant and ensures that the sample is the most probable one which is returned from database. The accession number for Pru p 3 was found as Q9LED1. The identified peptides are given in the Table 3.3.

Table 3.3: list of the peptides identified with their mass and sequence.

Start - End	**Mr (Da)**	**Mr (Da)**	**Mr (Da)**	**ppm**	**Miss**	**Sequence**
	Observed	Experimental	Calculated			
1-18	2007.97	2006.97	2006.99	-21	0	ITCGQVSSLAPCIPYVR
19-32	1385.62	1384.62	1384.63	-25	0	GGGAVPPACCNGIR
33-39	800.43	799.42	799.43	-22	0	NVNNLAR
45-52	964.42	963.41	963.43	-24	0	QAACNCLK
53-72	1904.97	1903.97	1904.01	-23	0	QLSASVPGVNPNNAAALPGK
73-80	923.44	922.44	922.46	-23	0	CGVSIPYK
81-91	1151.55	1150.54	1150.57	-21	0	ISASTNCATVK

3.4.2 Purification and characterization of Tri a 14

Wheat bran is naturally low in sugar while high in lipids. Therefore, a thorough defatting was performed with hexane to remove lipids to avoid the interaction of lipidic moieties with Tri a 14 resulting in adducts. Although the phenolic acids content of wheat bran is very high ranging from 3.3 - 3.9 mg/ g of bran, these are mostly present in the bound form (2.5-5.4 fold to extractable) and are not released even during 80% methanol extraction (Kim et al., 2006). Therefore, a level of 3% of PVPP was determined to be enough to adsorb the soluble phenolic acids.

3.4.2.1 Ion exchange chromatography

Wheat extract was made in deionised water, thus was low of ionic strength. The cation exchange resin, Streamline (Pharmacia, Uppsala, Sweden) removed non-cationic proteins from the mixture and eluted cationic proteins in a step gradient. The chromatogram (Fig. 3.9 a) represents two major peaks, the first broad peak containing unbound material while other contained bound proteins eluted by applying step gradient. SDS-PAGE of the bound fraction (Fig. 3.9 b) showed the presence of a wide range of proteins from 97 -7 kDa.

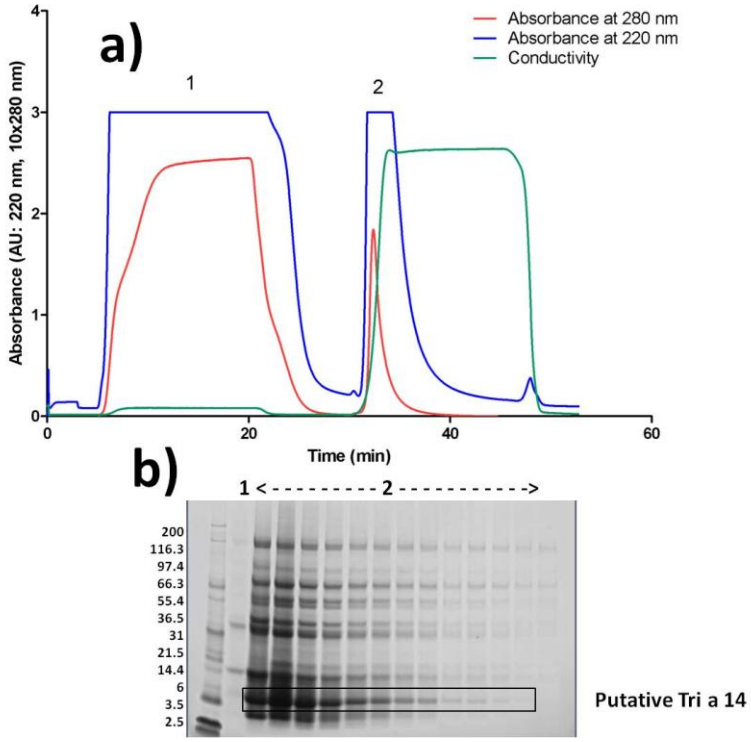


Fig.3.9: Ion exchange chromatography of putative Tri a 14 (a) and SDS-PAGE (b) of the bound fractions represented as peak 1 and 2.

3.4.2.2 Gel filtration

The gel filtration of putative Tri a 14 removed major high molecular weight contaminants by resolving the mixture of proteins into seven peaks (Fig. 3.10). The pooled fractions as identified on SDS-PAGE contained a mixture of three major proteins. The elution volume for putative Tri a 14 was found to be 91 mL which corresponds the protein of Mr ~ 9 kDa. The elution volume of Tri a 14 was unexpectedly a little more than that of Pru p 3 which may be due to differences in surface charge characteristics of wheat LTP made up with 9 basic and 7 acidic residues as compared to Pru p 3 with only 8 basic and 1 acidic residue resulting in bringing more water molecules (Palacin et al., 2007).

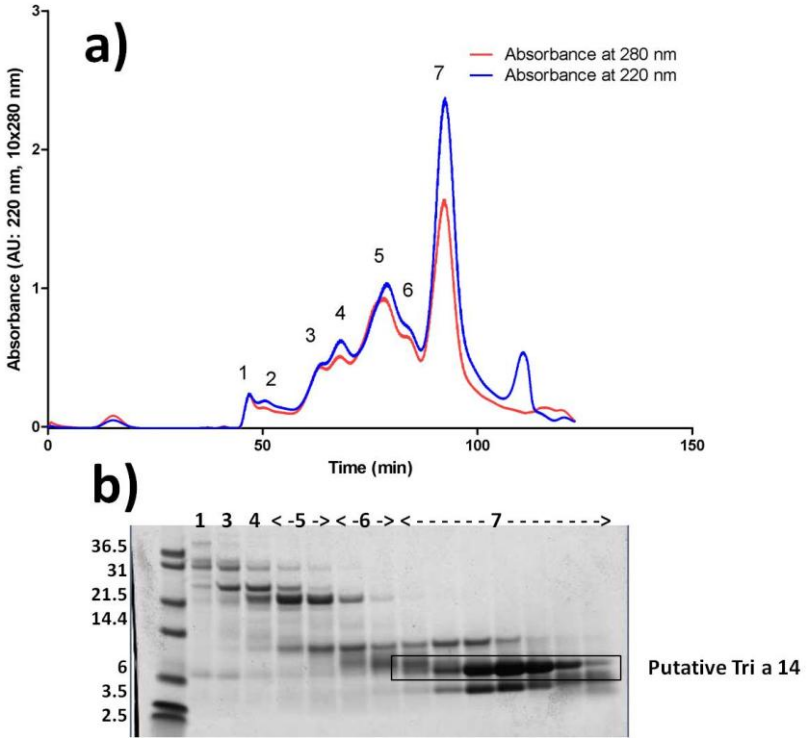


Fig.3.10: Gel filtration chromatogram (a) and SDS-PAGE of the peaks identified (b).

3.4.2.3 High resolution ion exchange chromatography

The pooled fractions obtained after gel filtration were subjected to high resolution ion exchange chromatography to remove contaminants co-eluting with Tri a 14. The chromatogram for this separation is given in Fig. 3.11.

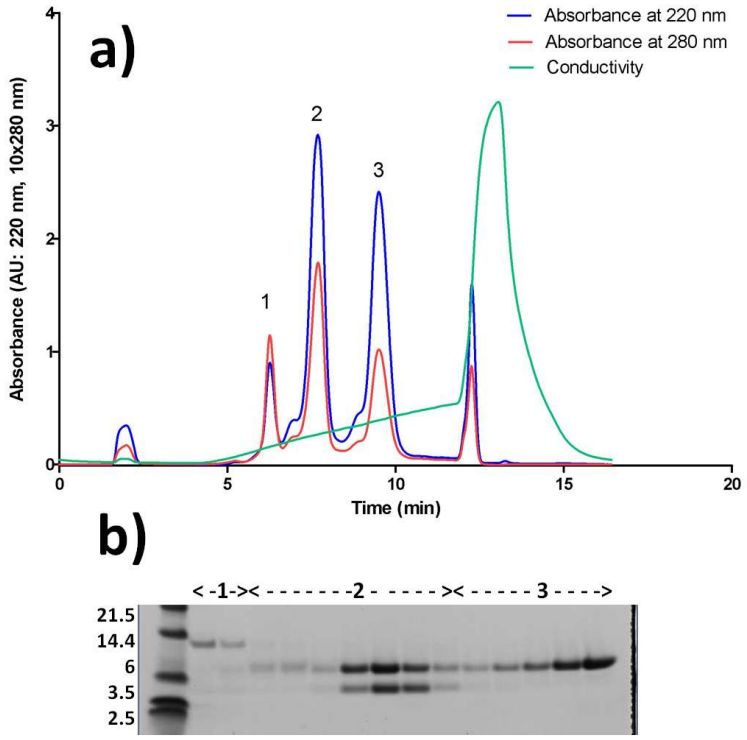


Fig. 3.11: High resolution ion exchange chromatography of the peaks obtained after gel filtration. (a) Represents the chromatogram while (b) represents the SDS-PAGE of the peaks marked on chromatogram.

The chromatogram consists of three peaks namely 1, 2 and 3, of which 2 and 3 are major peaks. SDS-PAGE of these fractions revealed that peak 1 consists of a protein with a molecular weight of 14 kDa and was thus assumed to be a contaminant. Peak 2 contained two abundant proteins in the fractions with molecular weight 7 and 9 kDa. Peak 3 also contained two proteins of molecular weight 9 and 7 kDa with the 9 kDa protein being more abundant. Therefore, the proteins obtained required further purification which was performed by hydrophobic interaction chromatography (section 3.4.2.4).

3.4.2.4 Hydrophobic interaction chromatography

Since peaks 2 and 3 obtained after high resolution ion exchange chromatography both contained 9 kDa proteins, both of them were subjected to hydrophobic interaction chromatography. Pooled fractions comprising peak 2 were saturated with ammonium sulphate which precipitated major portion of the protein leaving very little amount in supernatant. This supernatant was subjected to hydrophobic interaction chromatography and the chromatogram is given in Fig. 3.12.

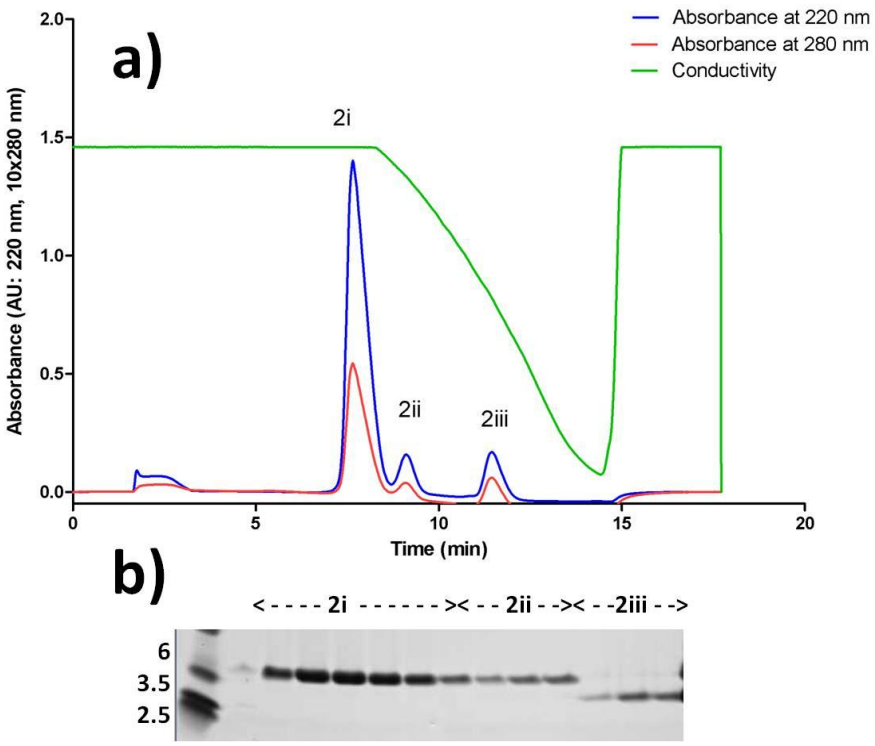


Fig. 3.12: Hydrophobic interaction chromatography of peak 2 obtained in high resolution ion exchange chromatography of putative Tri a 14. (a) represents the chromatogram while (b) represents the SDS-PAGE of the corresponding peaks.

Hydrophobic interaction chromatography successfully resolved the mixture of three proteins in peak 2 into peak 2i, 2ii and 2iii. However, due to the precipitation of the most of the proteins of peak 2, only a very small amount of these proteins were obtained and were identified through mass spectrometry only.

Peak 3 from high resolution ion exchange chromatography was also subjected to hydrophobic interaction chromatography to remove the minor contaminant corresponding to molecular weight 7 kDa. The chromatogram of this separation is given in Fig. 3.13.

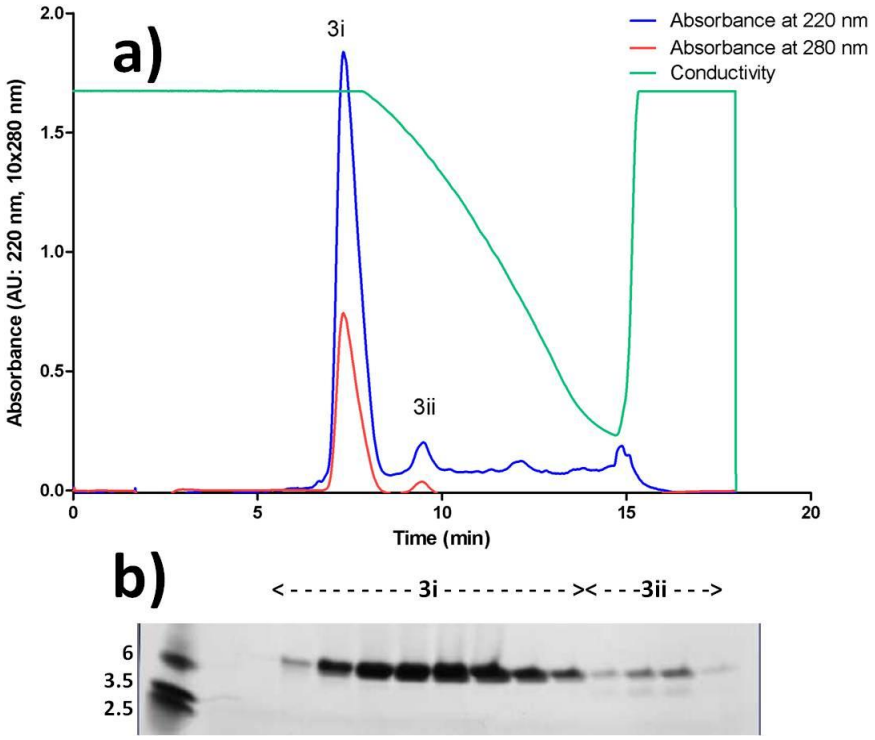


Fig. 3.13: Hydrophobic interaction chromatography of peak 3 (a) and SDS-PAGE of the peaks obtained (b).

Hydrophobic interaction chromatography of the peak 3 obtained in high resolution ion exchange chromatography (section 3.4.2.3) was resolved into two peaks namely 3i and 3ii with the major proportion of the protein Tri a 14 in 3i. This fraction was pooled and desalted by Sephadex G15 column followed by freeze drying and storage at -20°C. The yield of the protein is given in Table 3.4.

Table 3.4: Purification table for Tri a 14

Purification step	Yield	Step losses
Extract Preparation	2946 mg	-
Dialysis	-	-
Cation exchange chromatography	300 mg	2646 mg
Ultra filtration	ND*	-
Gel filtration	138 mg	162 mg
High resolution ion exchange chromatography	37 mg (as “peak 3”)	101 mg

3.4.2.5 Far UV CD

The protein secondary structure was determined by Far UV – CD according to the method described in section 2.1.4. The spectrum is given in Fig. 3.14.

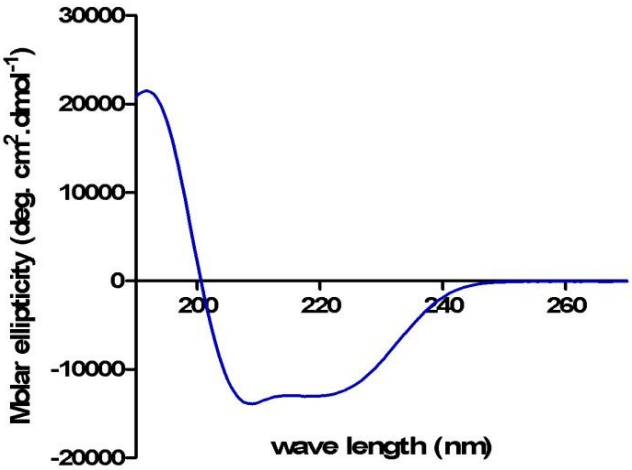


Fig. 3.14: CD spectrum of Tri a 14

The CD spectrum of Tri a 14 is similar to that of Pru p 3 and contains the same two negative peaks (minima) and a positive peak in the far UV region at the same positions. There was a difference observed between the two spectra which was the higher ellipticity of the minima at 208 nm. The spectrum indicates a similar folded structure to that of Pru p 3. The CD spectrum of Tri a 14 is very similar to those already published suggesting native folded protein was obtained after purification (Palacin et al., 2009).

The data was analyzed by using the same software CDPro and the proportion of α – helix and β – sheet along with turns and random coil were measured. Reference sets IBasis 3, 4, 6 and 7 were used by the program to calculate the structural components. The secondary structure fractions returned by the software are presented in Table 3.5.

Table 3.5: Structural components of Tri a 14

	CDSSTR					**ContinLL**			
	Helix	Sheet	Turn	Random		Helix	Sheet	Turn	Random
IBasis 3	0.41	0.15	0.17	0.26	**IBasis 3**	0.38	0.13	0.21	0.29
IBasis 4	0.38	0.17	0.18	0.27	**IBasis 4**	0.38	0.14	0.20	0.28
IBasis 6	0.40	0.14	0.16	0.31	**IBasis 6**	0.37	0.14	0.20	0.29
IBasis 7	0.40	0.13	0.16	0.31	**IBasis 7**	0.38	0.14	0.20	0.29

CDSSTR measured the α-helical structure of Tri a 14 as 40% while ContinLL measured it as 38%. The rest of the components were measured as 15% β-sheet, 17% turns, 28% random coil by CDSSTR while 14% β-sheet, 20% turns and 28% random coil by ContinLL.

3.4.2.6 Mass spectrometry

Tri a 14 was also characterized by MALDI – ToF – MS to determine the intact mass, indicate purity and confirm primary sequence. During purification, "peak 2" was further resolved by hydrophobic interaction chromatography into three different peaks namely 2i, 2ii and 2iii. These three peaks were analyzed by MALDI – ToF – MS. Peak 2i contained predominantly, a single protein with some minor contaminants as shown by MALDI spetrum (Fig. 3.15). The intact mass of the protein was found to be Mr 9467.

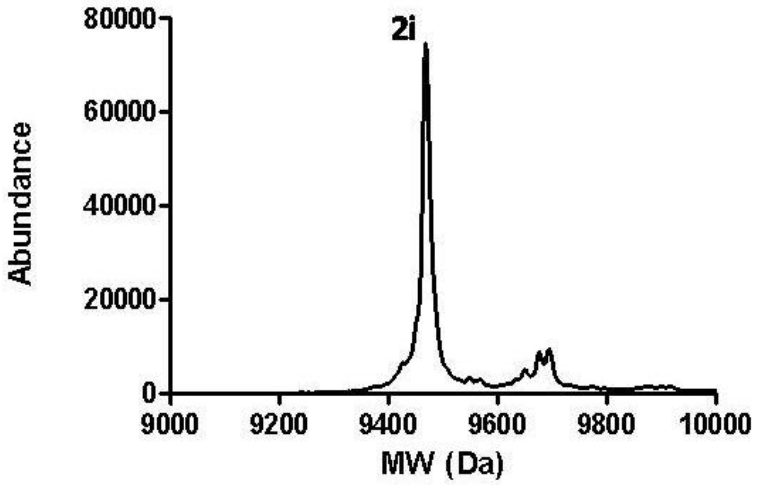


Fig 3.15: MALDI – ToF – MS of peak 2i.

To identify the sequence of this protein, in gel trypsin digestion followed by MALDI – ToF – MS identified 31 fragments as shown in Fig. 3.16 and in the Table 3.6. Major fragments identified were Mr 964, 1252 and 2075 Da. Remaining fragments were of low abundance.

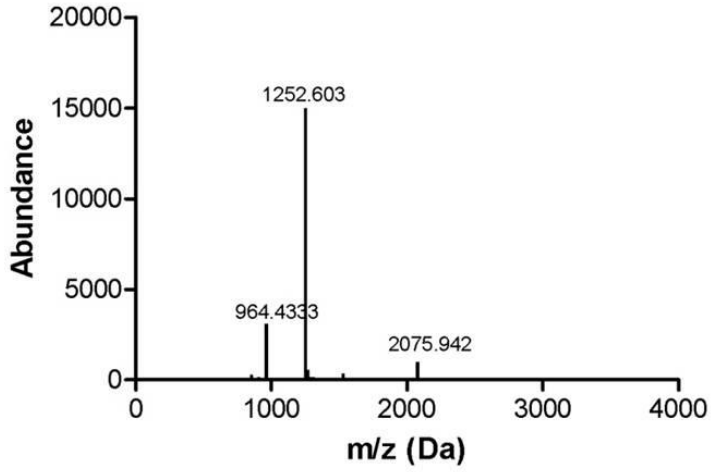


Fig. 3.16: MS spectrum representing peptide mass fingerprints of peak 2i protein

Mascot alogrithm searching returned several proteins with low probability based MOWSE scores with the highest at 57 which is not significant. Searching the database (SWISSPROT) for the lipid transfer protein from wheat with the similar intact mass to peak 2i returned LTP with accession number Q5NE29.

Table 3.6: Mass list of the peptides obtained after tryptic digestion of peak 2i.

m/z	Intens.	m/z	Intens.	m/z	Intens.	m/z	Intens.
712.12	89.68	**1072.52**	80.57	**1252.60**	15015.95	**1397.65**	57.51
832.31	75.59	**1088.70**	59.68	**1266.62**	123.51	**1527.71**	336.60
852.44	287.32	**1114.73**	60.99	**1270.62**	572.50	**2075.94**	1012.44
907.41	163.59	**1140.77**	71.08	**1280.62**	76.55	**2081.00**	43.10
947.40	86.76	**1206.60**	63.86	**1290.55**	136.73	**2089.94**	48.42
949.34	63.21	**1208.61**	78.31	**1309.62**	165.22	**2092.94**	39.19
964.43	3102.23	**1234.58**	79.98	**1314.53**	74.36	**2120.93**	31.33
980.44	83.57	**1237.59**	57.86	**1320.63**	125.88		

Peak 2ii contained two abundant mass events as shown in Fig. 3.17. Intact mass of the minor peak was found as 9467 (2iia). The major peak comprised two different proteins with intact masses of 9754 (2iib) and 9762 (2iic) Da. These three isoforms could not be separated by conventional methods used in this study and appeared as a single band on SDS-PAGE (Fig. 3.11 b). Therefore, in gel trypsin digestion followed by MALDI – ToF – MS of peak 2ii represented the MS spectrum of a combination of these three proteins. Searching MASCOT for the known proteins didn't return any significant match (highest MOWSE score 57).

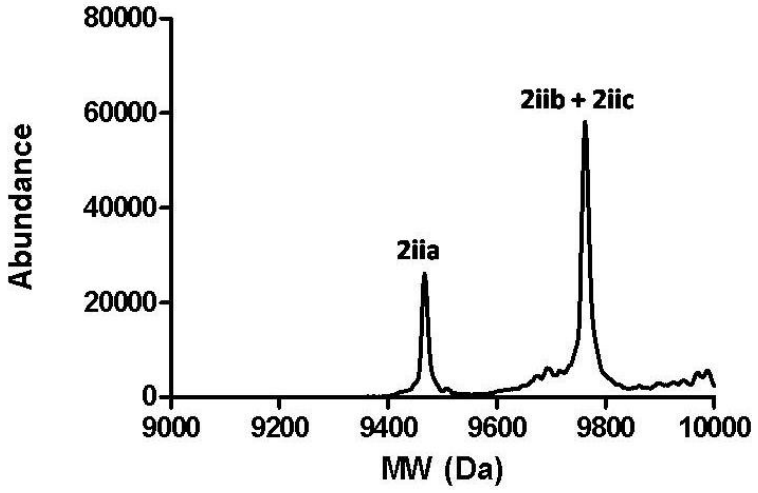


Fig. 3.17: MALDI – ToF – MS of peak 2ii.

The protein 2iia is the same intact mass as observed in protein 2i. The difference in mobility in hydrophobic column may be due to some structural changes during processing and running conditions. Searching database for intact masses (SWISSPROT) for peak 2iib (9754 Da) returned Q2PCB8 and Q2PCB7 with closed intact masses as known LTPs from wheat. Therefore, peak 2iib may be one of these two LTPs. In case of presence of Q2PCB8 (peak 2iib), it may be modified by N- glycosylation of lysine or O- glycosylation of serine or threonine during wheat bran extraction. In case of presence of Q2PCB7 (peak 2iib), it may be modified by α-cyano cinnamic acid from matrix used in MALDI – ToF – MS. Peak 2iic (9762 Da) appears to be an adduct of peak 2i with an additional mass of Mr 295. This additional adduct is the same as already reported with Tri a 14 (Douliez et al., 2001).

Peak 2iii was analyzed for its intact mass and purity by MALDI – ToF – MS. The MALDI – ToF – MS spectrum of this protein is given in Fig. 3.18.

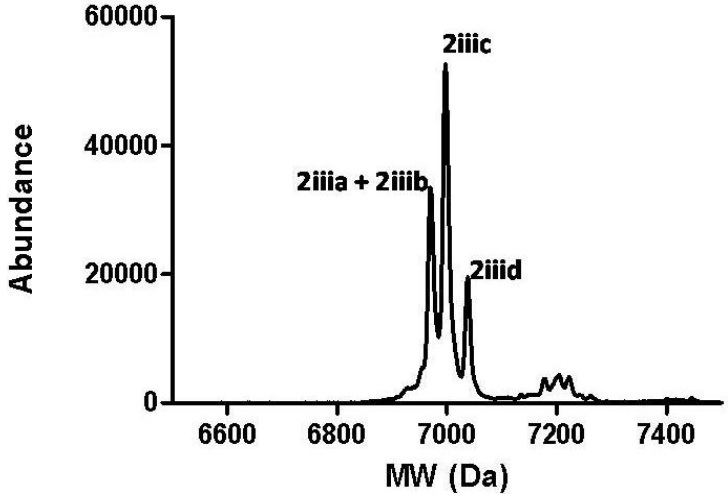


Fig. 3.18: MALDI – ToF – MS of proteins in peak 2iii

Peak 2iii contained four different proteins namely 2iiia (6969.78 Da), 2iiib (6971.5 Da), 2iiic (6996.7 Da) and 2iiid (7037.2 Da). Possibly, because this peak was the mixture of these four proteins, in – gel trypsin digestion followed by MALDI – ToF – MS of the digests returned an insignificant MOWSE score (highest MOWSE score 61). SWISSPROT database search for the intact mass of the proteins found in wheat returned Q5NE34 which may be either 2iiia or 2iiib because these two had similar masses. Protein 2iiic (6996.7 Da) remained unidentified while protein 2iiid (7037 Da) might be Q2PCC7 due to similar intact masses.

Peak 3 was well resolved in hydrophobic interaction chromatography (Fig. 3.12) and separated into two peaks namely 3i and 3ii. Peak 3i (putative Tri a 14) was analyzed by MALDI – ToF – MS and the spectrum obtained is shown in Fig. 3.19.

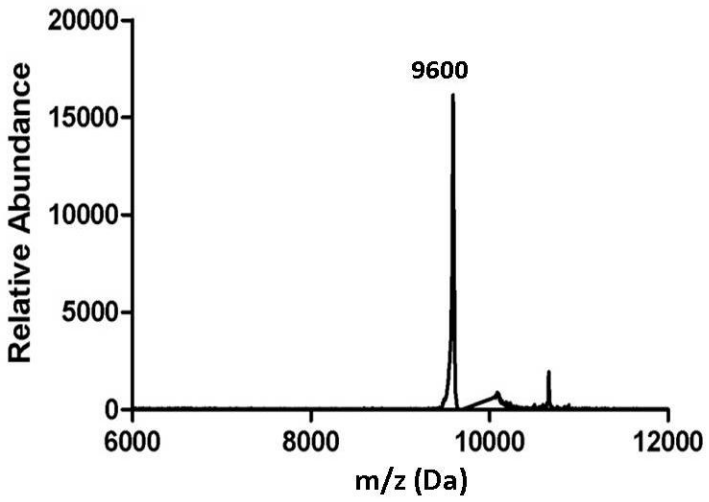


Fig. 3.19: MALDI – ToF – MS of Tri a 14.

The intact mass returned by the spectrum was Mr 9600 Da. The spectrum contains a single peak representing the purity of the sample. In gel trypsin digestion followed by MALDI – ToF – MS returned 43 fragments with Mr 852, 980, 1252 and 2081 Da as the major fragments. The list of peptides obtained is given in Table 3.7. The MS spectrum is given in Fig. 3.20.

Table 3.7: Fragments obtained after tryptic digestion of Tri a 14.

m/z	Intens.	m/z	Intens.	m/z	Intens.	m/z	Intens.
737.32	83.92	934.41	60.44	1252.60	16213.75	1995.95	35.02
806.44	57.18	963.40	144.47	1266.61	98.26	2081.02	1462.02
808.46	69.10	965.28	100.42	1270.62	258.49	2095.03	52.17
832.29	156.06	980.43	3275.26	1280.62	52.60	3395.04	26.06
837.37	56.44	991.43	81.42	1290.55	87.22	3416.54	38.82
852.44	16761.68	994.43	67.94	1309.63	299.33	3428.54	29.47
878.46	71.49	1178.59	148.91	1320.63	295.39	3474.57	689.22
903.46	68.29	1206.60	67.54	1397.66	55.83	3484.56	33.34
909.46	203.74	1208.61	82.72	1527.70	239.51	3487.57	47.33
920.47	286.71	1234.59	69.42	1965.91	99.62	3489.57	38.79
923.41	149.31	1236.58	67.64	1981.96	659.51		

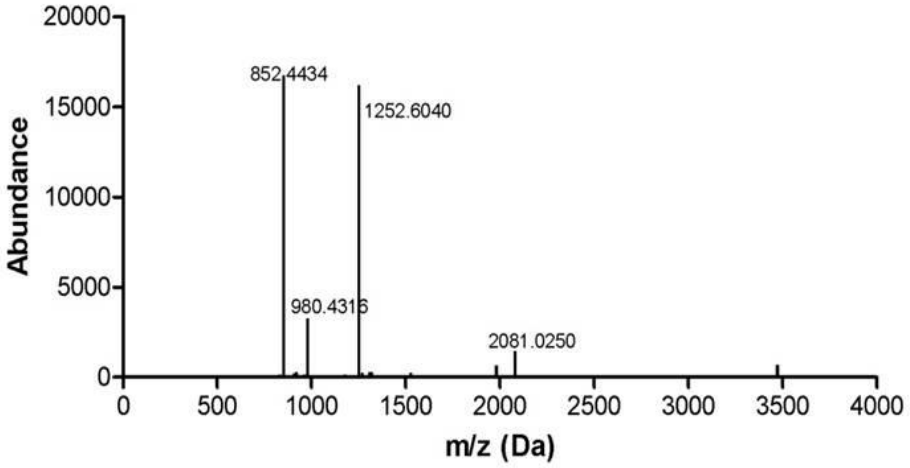


Fig. 3.20: MS spectrum representing peptide mass fingerprints of Tri a 14

The data was submitted to Mascot search engine to match with known proteins available in database. The search returned with protein accession number P24296 (Tri a 14) with a significant MOWSE score of 80. The identified peptides are given in Table 3.8.

Table: 3.8: List of the peptide fragments obtained after tryptic cleavage of Tri a 14

Start - End	**Mr** Observed	**Mr** Experimental	**Mr** Calculated	**ppm**	**Miss**	**Sequence**
33-40	852.44	851.44	851.44	0	0	NLHNQAR
45-52	980.43	979.42	979.42	3	0	QSACNCLK
57-67	1252.60	1251.6	1251.60	1	0	GIHNLNEDNAR
73-89	1981.96	1980.95	1980.94	5	0	CGVNLPYTISLNIDCSR
73-90	2081.02	2080.02	2080.01	5	1	CGVNLPYTISLNIDCSRV

Peak 3ii was in very low abundance for analysis with MALDI – ToF – MS. Therefore, in gel trypsin digestion followed by MALDI – ToF – MS was used to identify the protein. The probability based MOWSE score for this protein was insignificant. The best match was found to be the same protein found in peak 3i (P24296). The sequences of all proteins identified during purification are given in Fig. 3.21.

```
Q2PCB7 -ISCSTVYSTLMPCLQYVQQ-GGSPARGCCTGIQNLLAEANNSPDRRTICGCLKNVANGA 58
Q2PCB8 -ISCSTVYSTLMPCLQYVQQ-GGSPARGCCTGIQNLLVEANNSPDRRTICGCLENVANGA 58
Q9LED1 -ITCGQVSSSLAPCIPYVRG-GGAVPPACCNGIRNVNNLARTTPDRQAACNCLKQLSASV 58
Q5NE29 ALSCGQVDSKLAPCVAYVTGRASSISKECCSGVQGLNGMARSSSDRKIACRCLKSLATSI 60
P24296 -IDCGHVDSLVRPCLSYVQG-GPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGI 58
Q5NE34 --AC--EVGQLTVCMPAITT-GAKPSGACCGNLR----------AQQACFCQYAKDPSL 44
Q2PCC7 --AC--QASQLAVCASAILS-GAKPSGECCGNLR----------AQQPCFCQYAKDPTY 44

Q2PCB7 SGGPYITRAAALPSKCNVALPYKISPSVDCNSIH 92
Q2PCB8 SGGPYITRAAALPSKCNVALPYKISPSVDCNSIH 92
Q9LED1 PG-VNPNNAAALPGKCGVSIPYKISASTNCATVK 91
Q5NE29 KS-INMGKVSGVPGKCGVSVPFPISMSTNCDTVN 93
P24296 HN-LNEDNARSIPPKCGVNLPYTISLNIDCSRV- 90
Q5NE34 ARYITSPHARETLVSCGLAVPH-------C---- 67
Q2PCC7 GQYIRSPHARDTLQSCGLAVPH-------C---- 67
```

Fig. 3.21: Sequences of the proteins identified.

3.5 Chapter conclusion

The major LTP found in peach peel was found to be Pru p 3 while in wheat bran was Tri a 14. Pru p 3 has a higher pI (9.25) than Tri a 14 (8.25), which supported the selection of pH 6.5 for ion exchange chromatography of Pru p 3 and pH 5.5 for Tri a 14. The hydrodynamic volume of Pru p 3 was bigger than Tri a 14 making Pru p 3 run faster during gel filtration column than Tri a 14. This is due to the fact that Pru p 3 has a net positive charge of 7 while for Tri a 14, it is only 2. Both the proteins were purified by using different and additional steps than those already reported in literature (Carnés et al., 2002, Gaier et al., 2008, Palacin et al., 2007). In case of purification of Pru p 3, initial dialysis step removed low molecular weight proteins below 3.5 kDa which might interfere during ion exchange chromatography since they might have larger charge distribution making them closely eluted with Pru p 3. Initially, the protein was fractionated by using conventional cation exchange chromatography to remove anionic proteins and high molecular weight proteins having low pI which eluted in the early phase of fractionation. This step made purification of Pru p 3 much easier during gel filtration having major bulk of unwanted proteins removed during ion exchange chromatography and remaining being removed during gel filtration. However, there was a truncated form of Pru p 3 being coeluted with Pru p 3 at both steps which was removed by using a high resolution ion exchange resin. At the end of these three purification steps, protein was found to be extra pure as evident by SDS-PAGE and MALDI – ToF – MS shoing a single band and an intact single peak corresponding to Pru p 3. The problem of high salt in the purified protein samples was overcome by using an additional step of chromatography called desalting in deionized water which successfully removed salts from protein sample. This step

ensured that freeze drying of the protein sample predominantly contained extra pure Pru p 3 free from any buffer salt. The quantity of the protein thus purified was 120 mg which was enough to carry out the experiments shown in this thesis.

In case of purification of Tri a 14, this LTP does not form a high proportion of the total proteins in the extract. Therefore, a high yield method was chosen using Streamline, having a very high capacity to bind cationic proteins. This step made the purification of high quantity of Tri a 14 much easy in an efficient way. The cationic mixture of proteins thus obtained were applied to gel filtration which successfully removed most of bulk of unwanted proteins excluding various isoforms of LTPs. The pooled fractions here contained nine different isoforms of LTPs which were further fractionated by means of high resolution ion exchange chromatography and hydrophobic interaction chromatography. High resolution ion exchange chromatography successfully isolated Tri a 14 from 7 kDa LTPs and coeluted with altered structure Tri a 14 which was successfully removed by hydrophobic interaction chromatography. This purified form of Tri a 14 was also high in salt which were removed again by desalting in water. The freeze dried protein samples thus obtained ecsured predominantly protein free from buffer salts. The quantity obtained here was 110 mg which was enough to carry out the expereiments shown in this thesis.

The molecular mass of unreduced Pru p 3 was determined by MALDI – ToF – MS as 9136 Da. This is in agreement with the already reported Pru p 3 in the data base (Mr 9144 Da, SWISSPROT accession number, Q9LED1) when all eight cysteine moleculecules are reduced (Δ8 Da). Therefore, the protein purified was isolated in its native intact unreduced form with all cystein molecules intact. MALDI – ToF – MS of peptides obtained after tryptic digestion showed masses corresponding to those with a probability of identification of 95% with Q9LED1. The CD spectrum of Pru p 3 was characteristic of α-helical structure and was in agreement with those LTPs already reported in literature (Gaier et al., 2008). Both the intact mass and secondary structure of protein ensured the isolation of right form of Pru p 3 as found in native tissues to simulate the behaviour of protein in simulated gastroduodenal compartments. In conclusion, the protein identified was Q9LED1 (Pru p 3) with the same characteristics and behaviour as already reported in literature (Gaier et al., 2008).

The wheat extract contained several isoforms of LTP including five Mr 9 kDa (Fig. 3.14, 3.16 and 3.18, 3.20) and four Mr 7 kDa proteins (Fig. 3.17, 3.20). A total of seven LTPs were identified in the extract (Fig. 3.20) containing all five Mr 9 kDa and two Mr 7 kDa proteins. Among these isoforms, Tri a 14 was best characterized with the biochemical properties already reported. The

molecular mass of Tri a 14 was determined by MALDI – ToF – MS as 9600 Da. This is in agreement with the already reported Tri a 14 in the literature (Palacin et al., 2007) and in the data base (SWISSPROT accession number P24296, Mr 9607 Da). This showed that protein was isolated in its unreduced form. MALDI – ToF – MS of peptides obtained after tryptic digestion showed masses corresponding to the same protein with a probability based MOWSE score of 80 while the proportion of the matched peptide was 50%. The CD spectrum of the protein was also similar to those reported in literature (Palacin et al., 2009). Therefore, the protein was isolated in its native unreduced intact form as found in native tissues to simulate the experimental conditions made in this study. In conclusion, the protein isolated is the same as found in the database (P24296).

4 Development of transgenic wheat expressing Pru p 3

4.1 Introduction

Transgenic plants are a powerful tool for producing large amounts of recombinant proteins for industrial and pharmaceutical uses (Austin et al., 1994). These methods provide the advantages of provision of natural organs for protein storage, being easy to scale up and a low initial cost of growing the plants (Whitelam et al., 1993). Some factors are considered in making a transgenic plant which affect the protein production. One factor is the silencing of gene which may be prevented by using plants with single copy of rDNA, avoiding repetitive homologues sequences, selection of stable rDNA expression and use of lowest possible germplasm methylation activity (Kusnadi et al., 1997).

Transgenic plants have been made which have an increased yield as a consequence of improved photosynthetic processes, the manipulation of chloroplast functionality, modifying the quantity of enzymes responsible for sugar and starch metabolism, incorporation of farnesyl transferase and isopentenyl transferase as anti-senescence enzymes and over expression of mannitol, trehalose and gulatamete dehydrogenase to improve stress related response (Dunwell, 2000). Transgenic plants may also be used as factories to produce very large amount of proteins having pharmaceutical importance such as α-interferon, human serum albumin, glucocerebrosidase, hirudin. These methods may be regarded as safer than those employed from animal origin with a potential of contamination of human pathogens (Giddings et al., 2000). *Bacillus thuringiensis*, a well known source of plant herbicidal proteins and related genes had been in use to control the pests in plants by producing (*Bt* toxin) toxins activated into the pest midgut. The gene responsible for production of these toxin has been successfully cloned into the plants such as tobacco, tomato and potato and prevented against the related pests (Estruch et al., 1997).

The process of introduction of a target gene into a plant of interest is known as trans-formation. Usually, the target gene is transferred along with a selectable or screenable marker to distinguish between the transformed and non-transformed cells. The genetic construct or plasmid typically contains a promoter, a transgene with a screenable marker and a terminating signal. In one method of transformation, physical particles such as gold are coated with these plasmids and bombarded onto plant tissues and stable transformants are screened through the screenable marker usually encoding a protein that performs a chemical reaction resulting in a visible product (Christou, 1996, Fromm et al., 1986).

Transgenic material may have adverse effects on the environment. Therefore, there is a need of identification of potential hazards of genetically modified material on environment, human and animal health. The risk assessment thus involves assessment of substantial equivalence of the transgenic material to existing native material at various steps prior to obtaining the level of acceptance. Three scenarios are likely present: either fully equivalent or equivalent except for the inserted trait or non-equivalent. In the first scenario, no further assessment is required since the material is equivalent to the native material. In the second scenario, the assessment involves the tests focussing only the foreign genes and associated expression products such as phenotype, gross composition, allergenicity, nutrients, anti-nutrients and toxins. In the third scenario, the plant material needs to be assessed case by case for its characteristics. The safety assessment of transgenic plant materials include safety evaluation of recombinant proteins expressed, allergenicity, DNA analysis, gene expression analysis, proteomics, chemical fingerprinting, assessment of marker genes, detection and characterization of unintended effects and safety evaluation of whole material. Recombinant protein is assessed by means of electrophoresis of native and trypsinated forms, immunoreactivity, post-translational modifications, sequence and bioactivity. Safety assessment of whole transgenic material is carried out by feeding the material in appropriate ratios to animals such as rats. The allergenicity is assessed by molecular weight, resistance to gastric proteolysis, stability to heat and acid, glycoprotein test, induction of IgE response and presence of intact form in blood stream after oral ingestion. The disruption, silencing or modification of genes may result in formation of new metabolites or altered levels of expression which is hard to monitor because of limitation in knowledge of modification. Localization and characterization of inserted gene is also a part of risk assessment associated with transgenic material. Gas chromatography (GC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy are the most frequent techniques to map the chemical profile of the plant material (Magana-Gomez and Barca, 2009, Kuiper et al., 2001, Wendy Craig, 2008).

The influence of the food matrix may be an important factor affecting the digestibility of food allergens and hence affecting exposure of the immune system to immunologically active forms of allergens, or target genes in GMOs. This may be demonstrated by taking the example of highly allergenic foods, such as tree nuts and peanuts, which contain high level of fats which may protect allergenic proteins during digestion (Wijk et al., 2005, Lehrer et al., 2002, Teuber, 2002) or fruits such as peach (Fernández-Rivas et al., 2003) having very high proportion of water making allergens release at a faster rate than those in bread and hence more accessible to immune system in short

amount of time (hypothesis of this work). It has been shown that purified peanut allergens did not show as severe immune response as compared to the peanut extracts containing the same allergens although the stability of these allergens was unaffected by the presence of food matrix (Wijk et al., 2005).

The food matrix may affect the susceptibility of a protein to digestion in various ways. The irrelevant proteins and non-protein components may also alter the digestibility of and allergen or a target protein in a genetically modified plant by inhibiting proteases, acting as competing protease substrates or, if they have enzymatic activity, modifying them. A summary of the potential modifications and its effect on protein digestion is given in Fig 4.1.

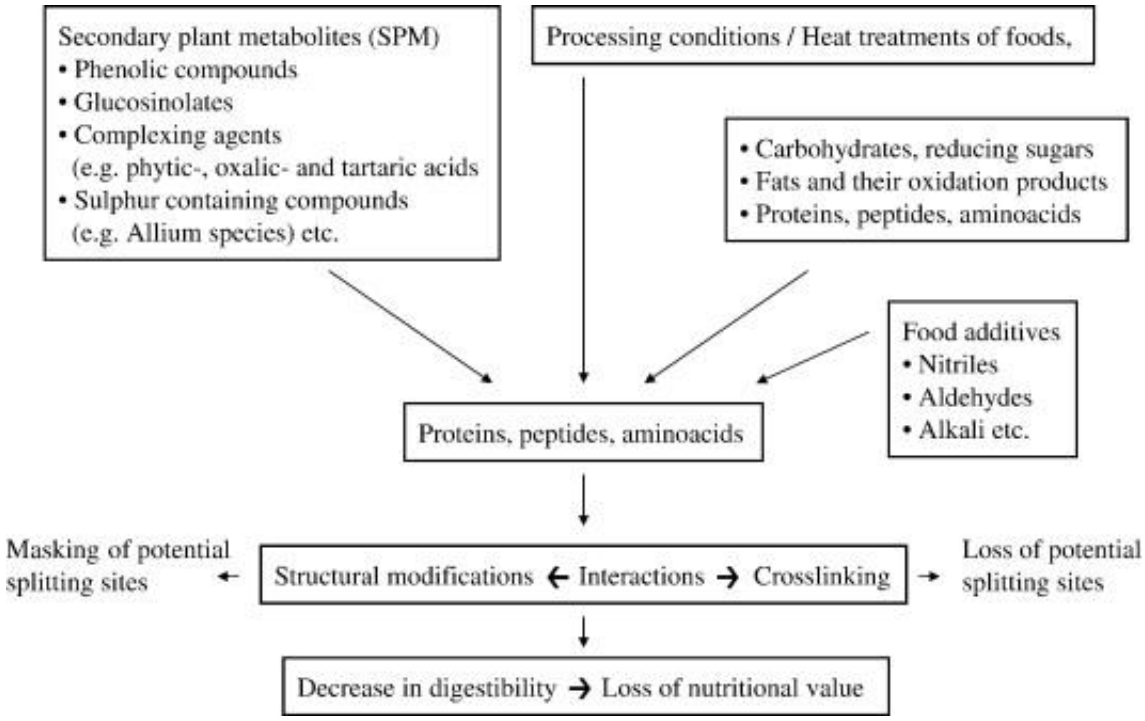


Fig. 4.1: Effect of non-protein components of food matrix on digestibility of proteins including allergens and target proteins in genetically modified plants. Reprinted by the permission from (Rawel et al., 2007).

Similarly, pectin has also been shown to decrease the digestibility of allergens by interfering with the viscosity of the digestion medium as well as the molecular mobility of allergen and pepsin within the medium (Polovic et al., 2007). Food matrix contains many components that may act as ligand for proteins. The effect of ligand binding on decreasing protein digestibility has been shown in literature (Smith and Wilson, 1991, Mandalari et al., 2009b). Similarly, interaction with native polyphenols also hampered the digestion of buckwheat groat proteins (Skrabanja et al., 2000) whilst interactions with components such as phytic acid commonly found in cereals reduced the digestion

of casein, a cows milk allergen (Lathia et al., 1987). Interaction of protein with lipids also has been shown to protect against digestion *in vitro* (Moreno et al., 2005). Interaction of lipids with proteins in emulsions initially enhanced the digestibility of intact protein and formed stable fragments which were resistant to further digestion *in vitro* (Macierzanka et al., 2009). In addition to the matrix itself, food processing can also modify proteins. On example of such a modification is the Maillard reaction. For example, ovalbumin, an allergen from egg was Maillard modified and it was shown that the new modified allergen had enhanced T-cell immunogenecity (Ilchmann et al., 2010). Glycation has also been shown to prevent the gastroduodenal digestion of beta-lactoglobulin (BLG) (Corzo-Martínez et al., 2010).

The investigation of the effect of the food matrix may be carried out in various ways such as either mixing in other matrices, making a starch gel or using a fluid system such as a viscous paste, a brittle gel or a hard solid system such as uncooked cereals. In the present study, we attempted to express Pru p 3 in wheat seeds to present Pru p 3 in an alternative physicochemical environment with a view to assessing the effect on its susceptibility to digestion.

4.2 Methods

4.2.1 Materials

All the chemicals used were of analytical grade. Nunc Maxisorp 96 well microtitre plates were obtained from Inter-med (MaxiSorp^{TM}, Thermo Scientific, Loughborough, Leicestershire, UK). Sodium dihydrogen ortho-phosphate was obtained from BDH, Leicestershire, UK. Sodium chloride was supplied by Riedel de Haën (Seelze, Germany). Disodium hydrogen phosphate, sodium chloride, potassium dihydrogen phosphate, potassium chloride, Tween 20, alkaline phosphatase conjugated anti – rabbit IgG raised in goat, horse radish peroxidise conjugated anti – rabbit IgG raised in goat, Sigma FAST BCIP/NBT (5 – Bromo – 4 – Chloro – 3 – indoyl phosphate/ nitro blue tetrazolium tablets), orthophenylene di-amine di-hydrochloride (OPD), 3,3',5,5', tetramethylbenzidine (TMB) were obtained from Sigma, Dorset, UK. Skimmed milk powder was obtained from a local supermarket. Sinapinic acid, trifluoroacetic acid, dithiothreitol (DTT) and acetonitrile were of analytical grade and were obtained from Sigma, Dorset, UK. Standard MALDI target plates were obtained from Bruker Daltonics, Coventry, UK. The rabbit anti Pru p 3 (IgG) was kindly provided by Dr. Philippe Delahaut, CER Group, Europe.

4.2.2 Development of Pru p 3 construct

Two genes namely Phil Johnson-Syed Abdullah 1 (PJSA1) expressing Pru p 3 extracellularly and Phil Johnson – Syed Abdullah 2 (PJSA2) expressing Pru p 3 with –KDEL at its C-terminus retaining this protein at endoplasmic reticulum were purchased from GENEART (GENEART AG, Regensburg, Germany). Briefly, the synthetic gene PJSA1 was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into plasmid pMA (ampicillin resistant) using KpnI and SacI cloning sites. The plasmid was inserted into the *E. coli* (DH5α) and grown in an ampicillin containing medium. The plasmid DNA was purified from the transformed bacteria and the concentration determined by UV absorbance at 260 nm. The final construct was verified by sequencing.

The development of the construct was carried out by Phil Johnson at IFR, Norwich Research Park, Norwich, UK. The purchased gene was inserted into cloning vector pLRPT (kindly provided by Huw Jones and Peter Shewry, Rothamsted Research, Harpenden, UK) which has the wheat high molecular weight glutenin promoter 1Dx5 for expression of the gene (Lamacchia et al., 2001). The pLRPT was digested with BamH1 and Nco1 restriction enzymes and ligated with PJSA1 or PJSA2 according to manufacturer's instructions. Analysis using agarose gel electrophoresis was conducted to verify the effectiveness of the ligation reaction. The resulting plasmids were inserted into *E. coli* (DH5α) and the strain grown in an ampicillin containing medium. Finally, the cells of *E. coli* carrying the plasmids were harvested, and the plasmids isolated. The isolated plasmid was then sent to Rothamsted Research to insert them into the wheat (cv Cadenza).

4.2.3 Construction of transgenic wheat

Wheat transformation was performed by Caroline Sparks and Huw Jones at Rothamsted Research, Harpenden, UK. The construction was carried out as described by Sparks and Jones (Sparks and Jones, 2004, 2009). Twenty milligrams of gold particles (0.6 µm, BioRad Laboratories, Hertfordshire, UK) were suspended in 1 mL ethanol and sonicated to wash the particles. The step was repeated three times to assure cleanliness of the particles followed by washing with 1 mL of water at least twice. Finally, the particles were suspended in 1 mL water and vortexed to make homogenous suspension prior to taking 50 µL aliquot of which the aliquots were stored at -20°C until required for coating.

The gold particle suspension was thawed at room temperature, resuspended by sonication prior to adding 5 µL of plasmid preparations (PJSA1 or PJSA2, 1 mg/ mL in water). The mixture was vortexed briefly to assure homogenous mixing. Fifty microlitres of $CaCl_2$ (2.5 M) and 20 µL of spermidine (0.1 M) were added to the mixture and vortex mixed. The plasmid coated gold particles were recovered after centrifugation and washed with 150 µL of ethanol. Finally, the particles were suspended in 85 µL ethanol and kept on ice until bombardment.

A volume of 5 µL of the plasmid coated gold particle suspension was applied to a macro carrier membrane on a rupture disc and placed into gas acceleration tube of PDS-1000/ He particle gun (BioRad Laboratories, Hertfordshire, UK). The gun was fired under a vacuum of 91.4-94.8 kPa on wheat scutella containing embryo previously placed on petri dishes with induction medium. The scutella were incubated at 22°C in the dark for induction of callus for 3-5 weeks. After primary induction, calli were transferred to RZ + selection in 9 cm petri dishes with high lids and further incubated for 3-4 weeks. After this incubation, calli were transferred to regeneration medium in Magenta vessels without hormones. After this incubation, plantlets were transferred to soil and grown. Plants were screened for the presence of Pru p 3 gene by PCR.

4.2.4 Analysis of transgenic seeds

To analyze the transgenic seeds for the presence and level of expression of Pru p 3, immunoblotting and ELISA were employed. The dot blotting was employed to find the evidence of Pru p 3 presence while ELISA was employed to detect very low amount of Pru p 3 within the extract and to quantify them.

4.2.4.1 Analysis by ELISA

4.2.4.1.1 Development of ELISA

Two different primary antibodies raised against Pru p 3 in rabbit were kind gifts from Philippe Delahaut (CER Groupe, Département Santé, Marloie, Belgium) and used coded as M709 and M710. Both the antibodies were screened for optimal titre in such a way that highest possible dilution preparations used. Again, the plate coating concentration of protein, type of buffer for coating and suitable dilution of primary and secondary antibody (horse raddish peroxidise conjugated anti – rabit IgG produced in goat) were determined during development of the ELISA. The method used is described in section 2.4.2.

4.2.4.1.1.1 Selection of buffer

Optimum coating condition was determined using 0.5 µg/ mL Pru p 3 in either 50 mM carbonate/ bicarbonate buffer pH 9.62 or 9.5 mM phosphate buffer saline pH 7.32. Pru p 3 was dissolved in these buffers at a concentration of 0.5 µg/ mL and a volume of 200 µL of protein solution applied into each well of 96 well microtitre plate. The plates were coated overnight and the next day, the plate was washed and air dried. The ELISA was carried out with OPD as substrate as described in section 2.4.2.

4.2.4.1.1.2 Determination of optimal concentration of secondary antibody

One 96 well microtitre plate was coated with 200 µL/ well of 0.5 µg/ mL Pru p 3 in PBS over night at 4°C. Primary anitibody dilutions covering 1:1000 (v:v), 1:5000 (v: v), 1:10,000 (v: v), 1:50,000 (v: v), 1:100,000 (v: v), 1:500,000 (v: v), 1:1,000,000 (v: v), 1:5,000,000 (v: v) and 1:10,000,000 (v: v) were prepared and further diluted 1:1 with PBST prior to applying into plates. Secondary antibody diluted either 1:1000 (v: v) or 1:5000 (v: v) in PBST was applied to the plates and the procedure followed as described previously.

4.2.4.1.1.3 Determination of optimal coating concentration

To determine the optimal coating concentration, five different plates were coated with 0.2, 0.5, 1, 2 and 5 µg/ mL of Pru p 3 in PBS as described above. 100 µL of primary antibody (either M709 or M710) diluted as 1:100,000 (v: v) in PBST was mixed with 100 µL of PBST and applied into each well of plate and ELISA carried out as described with 1:5000 (v: v) dilution of secondary antibody and OPD as substrate.

4.2.4.1.1.4 Selection of antibody

There were two antibodies (IgG) supplied namely M709 and M710 against Pru p 3 in rabbit. Both antibodies were diluted in PBST in ratios ranging 1:1000 (v: v), 1:5000 (v: v), 1:10,000 (v: v), 1:50,000 (v: v), 1:100,000 (v: v), 1:500,000 (v: v), 1:1,000,000 (v: v), 1:5,000,000 (v: v) and 1:10,000,000 (v: v). 100 µL of this antibody solution was mixed with 100 µL of PBST and applied to the wells of 96 well microtitre plate coated previously with 0.5 µg/ mL Pru p 3 in PBS. ELISA was carried out as described previously with 1:5000 (v: v) dilution of secondary antibody and OPD as substrate.

4.2.4.1.1.5 Determination of optimal concentration of primary antibody

To obtain a reasonable absorbance with respect to primary antibody, the optimum concentration of antibody was determined. From the secondary antibody dilution determination experiment, the

suitable antibody dilution selected was either 1:500,000 (v: v) or 1:1,000,000 (v: v). Therefore, a serial dilution of Pru p 3 ranging 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001 µg/ mL in PBST was mixed with either 1:500,000 (v: v) or 1:1,000,000 (v: v) dilution of primary antibody in triplicate and applied to the wells of 96 well microtitre plate previously coated with 0.5 µg/ mL Pru p 3 as described earlier. ELISA was carried out with 1:5000 (v: v) dilution of secondary antibody and OPD as substrate.

4.2.4.1.1.6 Quantification of Pru p 3 in peach extract

To determine the levels of Pru p 3 in peach extracts, a calibration curve needs to be established. For this reason, standard dilutions of Pru p 3 were prepared as 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 and 0.000001 µg/ mL in PBST. Peach extract was prepared for peach peel and pulp separately. The peel and pulp were separated from the fruit by using a domestic knife. Immediately after removal, the peel or pulp was frozen in liquid nitrogen by immersing and stored at -80°C until required. The frozen peel or pulp was ground in liquid nitrogen in a waring blender and a frozen powder obtained after evaporation of nitrogen. Extraction buffer (50 mM phosphate buffer pH 7.0, EDTA 2 mM, 3 mM NaN_3 and 20 mM sodium diethyldicarbamate) was mixed with the powdered peel of pulp in 2:1 ratio (v: w). The suspension was stirred for on hour at room temperature. After extraction, the suspension was centrifuged at 1700 x g and supernatant retained while pellet discarded. One hundred microlitres of the extract or Pru p 3 standard was mixed with 100 µL of primary antibody and ELISA carried out with 1:5000 (v: v) dilution of secondary antibody and OPD as substrate as described previously.

4.2.4.1.2 Validation

4.2.4.1.2.1 Spike recovery of Pru p 3 from wheat matrix

"Spike recovery" was established in this study to investigate the effect of food matrix on the physical and chemical interaction between the analyte and matrix. In this experiment, the purified form of the analyte (Pru p 3 in this case) is spiked into the matrix and allowed to interact fully. At the end of interaction, the analyte is extracted from the matrix and observed whether the extractability of the analyte from the matrix is completely achieved by conventional methods of extraction or the matrix has interactions with analyte which becomes a hurdle in this extraction.

To investigate the effect of the wheat matrix on Pru p 3 extractability, purified Pru p 3 was "spiked" in wheat flour and re-extracted. For this reason, two wheat grains (cultivar variety "cadenza") weighed separately as 40.6 and 42.9 mg were pressed between pliers and fine powder obtained. To

each of these two samples, 1.635 µg of purified Pru p 3 was added and mixed well. The sample was left over night in a fume cupboard covered with aluminium foil to dry the flour. The next day, both samples were extracted with 342.8 and 343.2 µL of extraction buffer as described for peach peel extraction in 4.4.1.5. One of the samples was vortxed for one minute followed by sonication for 5 minutes and a final vortexing for 1 minute. The second sample was vortexed for two minutes only. The samples were centrifuged at 22100 x g and the supernatant retained for Pru p 3 quantification. ELISA was performed as developed earlier, except that the substrate for horse reddish peroxidase was TMB.

4.2.4.1.3 Analysis by ELISA

Eight seeds from each plant were selected for ELISA study. Each grain was cut into two halves making sure that embryo was undamaged. The embryo was kept stored in a 96 well microtitre plate and wrapped with cling film to make sure moisture does not affect the seed. The other half of the seed was pressed between two pliers to a fine powder and suspended in 50 mM phosphate buffer pH 7.0 (8x, w: v). The suspension was vortexed for one minute followed by sonication for five minutes and then vortexing again for one minute. The suspension was centrifuged and supernatant was used for ELISA assay. One hundred microlitres of the extract was mixed with 300 µL of PBST and ELISA was carried out as described in section 2.4.2.

4.2.4.2 Analysis by immunoblotting

4.2.4.2.1 Dot blot analysis

For dot blot analysis, a serial dilution of purified Pru p 3 either in water or in blank wheat (cv Cadenza) extracts as 2000, 666, 222, 74, 24.7, 8.23, 2.74, 0.9 and 0.3 ng/ µL was prepared. Samples from various transgenic batches were withdrawn randomly and given codes as given in Table 4.1. One grain selected was cut into two halves so that the embryo was undamaged. The embryo was kept stored in a lock cabinet while the other half was crushed between two pliers to make a fine powder. To the powdered material, 50 mM phosphate buffer containing 20 mM sodium diethyl dicarbamate pH 7.0 was added in 1:8 ratio (w: v). The suspension was vortexed for one minute followed by sonication for 5 minutes and a final vortex of 1 minute. The suspension was centrifuged and the supernatant was analyzed. On a nitrocellulose membrane, various horizontal lanes were created by putting dots of either standard in water or in wheat extract or transgenic sample with 1 µL volume. The membrane was dried at room temperature and blotting carried out as described in section 2.4.3.

4.2.4.3 Analysis by multiple reaction monitoring (MRM)

4.2.4.3.1 Development of MRM

For MRM development, a solution of 50 µM Pru p 3 was reduced and alkylated followed by digestion over night with trypsin as described in 2.3.3. Briefly, the LTP was reduced with 500 mM DTT at 95°C for 30 minutes followed by alkylation with 500 mM iodoacetamide. Prior to make dilutions for MRM analysis i.e generating the calibration curve, the sample was applied to MALDI – MS to investigate whether the reduction and full digestion of the protein occurred.

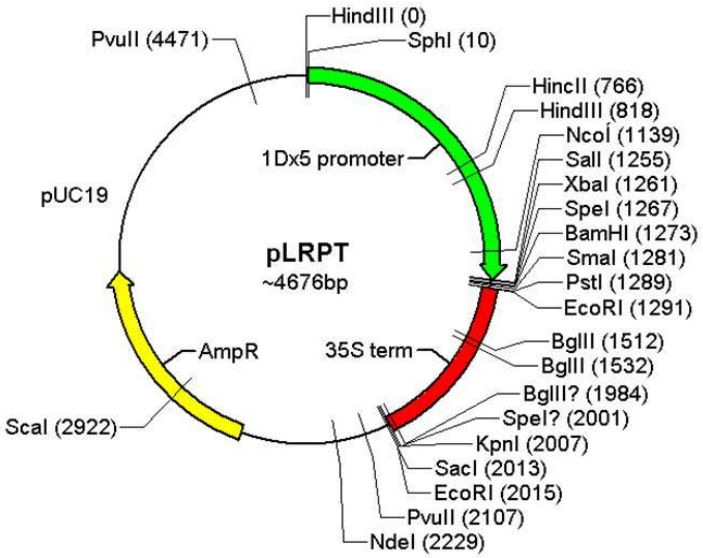
4.2.4.3.2 Digestion conditions

To investigate the digestibility of LTP required for MRM, the LTP was digested under various conditions such as reduction and alkylation of LTP under mild (100 mM of both DTT and iodoacetamide) and harsh conditions (500 mM of both DTT and iodoacetamide). Initially, the protein was digested with trypsin overnight to get enzyme specific fragments for analysis. After digestion, the sample was analyzed with MALDI – ToF – MS to check whether the protein was digested fully into small fragments.

4.3 Results

4.3.1 Development of the clone

The structure of pLRPT along with the sequences of Pru p 3 with signal peptide, PJSA1 and PJSA2 are given in Fig 4.2.



pLRPT cloning vector contains: 1Dx5 promoter ~1250bp
35S terminator ~710bp
in pUC19 backbone
(plus part of multi-cloning site from pBluescript at 3' end of 35S terminator)

```
PJSA1    ATGGCTTACTCTGCCATGACTAAGCTTGCTTTGGTGGTGGCCTTGTGCATGGTTGTGAGC 60
PJSA2    ATGGCTTACTCTGCCATGACTAAGCTTGCTTTGGTGGTGGCCTTGTGCATGGTTGTGAGC 60

PJSA1    GTGCCCATTGCTCAAGCCATAACATGTGGCCAAGTGTCCAGCAGCCTTGCACCATGCATA 120
PJSA2    GTGCCCATTGCTCAAGCCATAACATGTGGCCAAGTGTCCAGCAGCCTTGCACCATGCATA 120

PJSA1    CCCTACGTGAGAGGCGGTGGAGCTGTGCCTCCAGCTTGCTGCAACGGCATTAGGAACGTC 180
PJSA2    CCCTACGTGAGAGGCGGTGGAGCTGTGCCTCCAGCTTGCTGCAACGGCATTAGGAACGTC 180

PJSA1    AACAACTTGGCAAGGACCACCCCTGACCGCCAGGCCGCTTGCAACTGCCTGAAACAGCTT 240
PJSA2    AACAACTTGGCAAGGACCACCCCTGACCGCCAGGCCGCTTGCAACTGCCTGAAACAGCTT 240

PJSA1    TCCGCCAGCGTCCCCGGAGTCAACCCTAACAATGCCGCAGCGCTTCCCGGAAGTGTGGAG 300
PJSA2    TCCGCCAGCGTCCCCGGAGTCAACCCTAACAATGCCGCAGCGCTTCCCGGAAGTGTGGAG 300

PJSA1    TTAGTATTCCTTACAAGATTAGCGCCTCCACCAACTGCGCCACCGTGAAG-----TGA-- 353
PJSA2    TTAGTATTCCTTACAAGATTAGCGCCTCCACCAACTGCGCCACCGTGAAGAAGGACGAGC 360

PJSA1    --
PJSA2    TC 362
```

Fig 4.2: Initial structure of pLRPT before cloning PJSA1 or PJSA2 into it. The sequences of both the genes are also given.

The gene PJSA1 encodes wild type Pru p 3 and thus has a stop codon "TGA" after the 350 bp sequence of Pru p 3 gene. PJSA2 is the gene encoding wild type Pru p 3 with an additional peptide

"KDEL" sequence at the N-terminus which is responsible for its retention in the endoplasmic reticulum. Therefore, the 350 bp gene has additional gene "AAGGACGAGCTC" at its end without any stop codon. Agarose gel electrophoresis was used to monitor the development of the clone (Fig. 4.3) which confirmed the successful cloning of the gene into the vector. This is demonstrated by the fact that after digestion with restruiction enzymes, the vector a large proportion of gene which is replaced by the Pru p 3 after ligation. Therefore the net effect on the vector is its reduction in size, which is indicated by its greater mobility in agarose medium.

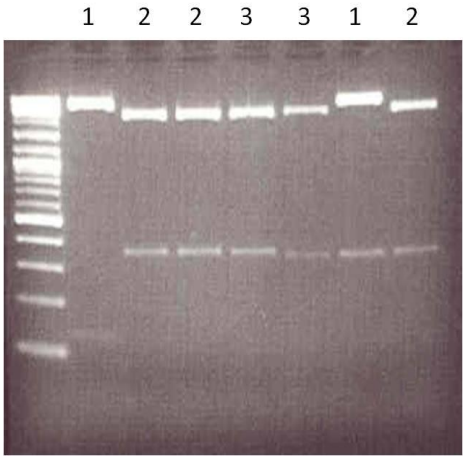


Fig 4.3: Agarose gel electrophoresis to confirm the insertion of PJSA1 and PJSA2 genes into pLRPT. 1) pLRPT, 2) pLRPT-PJSA1, 3) pLRPT-PJSA2.

4.3.2 Wheat transformation

Rothamsted Research successfully generated transgenic plants as demonstrated by the PCR experiments through which, the Pru p 3 constructs were identified in the transgenic plants.

4.3.3 Analysis of transgenic seeds

4.3.3.1 Analysis by ELISA

ELISA was established to quantify the levels of Pru p 3 expressed into the transgenic wheat constructs. ELISA has been commonly employed to either measure the quantity of allergen (Carnés et al., 2002, Duffort et al., 2002, Palosuo et al., 1998, Luczynska et al., 1989) or specific antibodies (Lombardero et al., 2004) in to the extracts and other samples or to investigate the cross-reactivity between the proteins (Asero et al., 2002). ELISA is a simple way to determine the levels of Pru p 3 within small samples such as single seed of wheat grain without purifying them. In case of wheat seeds being hexaploid in nature, there is a need of analyzing the seed with keeping the embryo of

the same seed safe for further growth. Therefore, the volume of sample drops to half seed with a need of an optimum method of extraction for analysis.

4.3.3.1.1 Development of ELISA

Before screening the transgenic seeds, an ELISA was set up to determine the quantities of Pru p 3 within the samples and the suitable range of dilutions within the quantification range of ELISA developed. Many factors were optimized here such as buffer, coating concentration, primary antibody and secondary antibody.

4.3.3.1.1.1 Selection of suitable buffer for coating

The buffers used for coating were PBS and carbonate buffer. After comparison between the plates, it was observed that carbonate/ bicarbonate buffer coated plates showed a very high back-ground as compared to PBS. This would interfere with the sensitive absorption profile of ELISA. Therefore, for all analysis and further studies, microtitre plates were coated with Pru p 3 in PBS.

4.3.3.1.1.2 Determination of optimal concentration of secondary antibody

Two different dilutions of secondary antibody were used, 1:5000 (v: v) and 1:1000 (v: v) in PBST. On comparison between the two dilutions of secondary antibody, the difference between the absorbance values were not very distinct. A very small difference was observed between the absorbance values at various dilutions of primary antibody. Therefore, the economic dilution 1:5000 (v: v) was selected for the rest of the study. The graph is given in Fig.4.4.

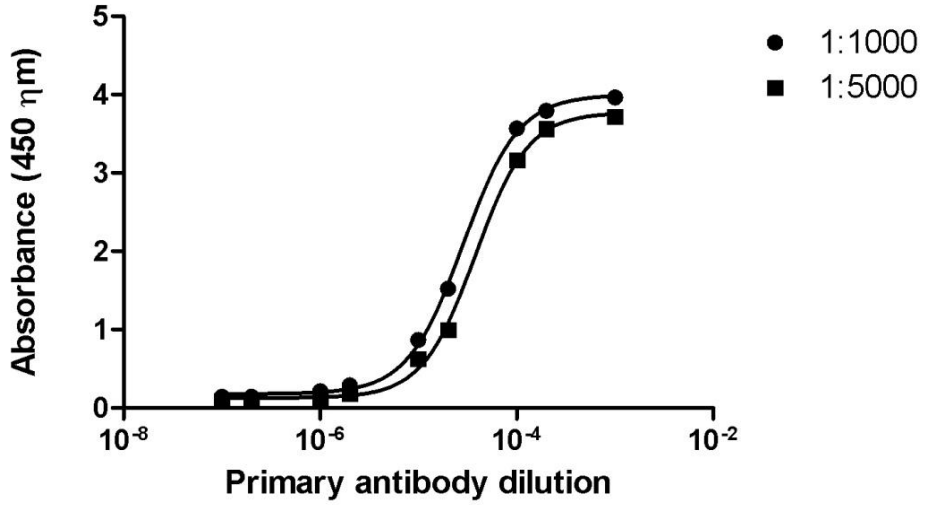


Fig.4.4: Dose response of primary antibody (M710) to ELISA. Each point in the curve represents mean of three individual observations within a single plate. 1:1000 and 1:5000 represent secondary antibody dilution (v: v) in PBST applied in the same plate.

4.3.3.1.1.3 Optimization of coating concentration

All five chosen concentrations for plate coating showed similar absorbance except for 0.2 µg/ mL which showed slightly lower absorbance. This showed that 0.2 µg/ mL concentration of Pru p 3 leaves very little gaps in coating and hence is not suitable for coating. Among the rest four concentrations, 0.5 µg/ mL concentration was minimum concentration which showed equivalent absorbance with respect to the rest. Therefore, for the rest of the experiments, the coating concentration of 0.5 µg/ mL was chosen. The graph of comparison among various concentrations for both primary antibodies is given in Fig. 4.5.

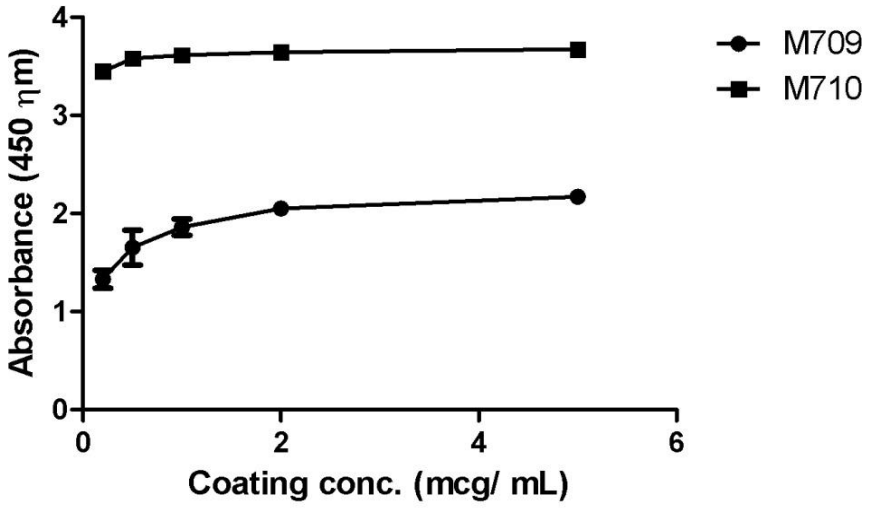


Fig.4.5: Comparison of the plates coated with five different concentrations of Pru p 3 solution. Both the primary antibodies (M709 and M710) were diluted to 1:500,000 (v:v) in PBST while secondary antibody was diluted to 1:5000 (v:v) in PBST.

4.3.3.1.1.4 Selection of antibody

There were two primary antibodies supplied, M709 and M710. Selection of antibody for the rest of the experiment was made by conducting ELISA as described earlier. Both the antibodies were applied in standard dilutions and absorbance recorded after substrate application. On comparison between the two primary antibodies, it was found that M709 produced lower absorbance for the same dilution as for M710. This implies that serum M709 contained smaller amount of IgG than M710. Therefore, for the rest of the experiments, antibody M710 was selected. The graph for the absorbance valuess are given in Fig. 4.6.

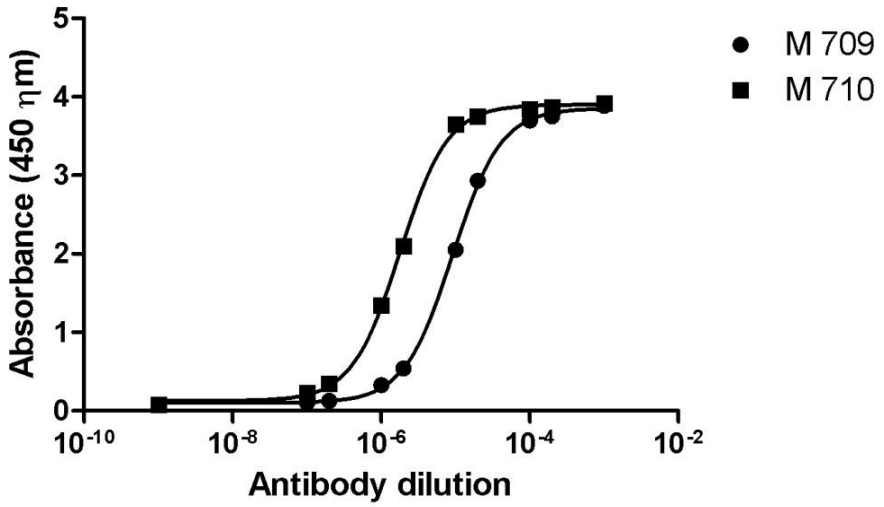


Fig.4.6: Antibody dilution curves of two antibody preparations (M709 and M710) on the same ELISA plate. Secondary antibody was diluted to 1:5000 (v: v) in PBST.

4.3.3.1.1.5 Determination of optimal concentration of primary antibody

From the data obtained in Fig. 4.5, two dilutions were selected to optimize the primary antibody dilution concentration. ELISA inhibition was carried out with Pru p 3 standards prepared as described previously. The dose response graph obtained for the absorbance after applying substrate is given in Fig. 4.7. On comparing the two dilutions, it was found that dilution 1:500,000 (v: v) produced better response for the standards while keeping the absorbance higher than dilution 1:1,000,000 (v: v). Therefore, for the rest of the experiments, this dilution was used throughout the study.

From the dose response, it is clear that ELISA established has a quantification range on 10 ηg/ mL – 10 µg/ mL. The method developed showed a very high reproducibility and showed equivalent quantifications for given samples.

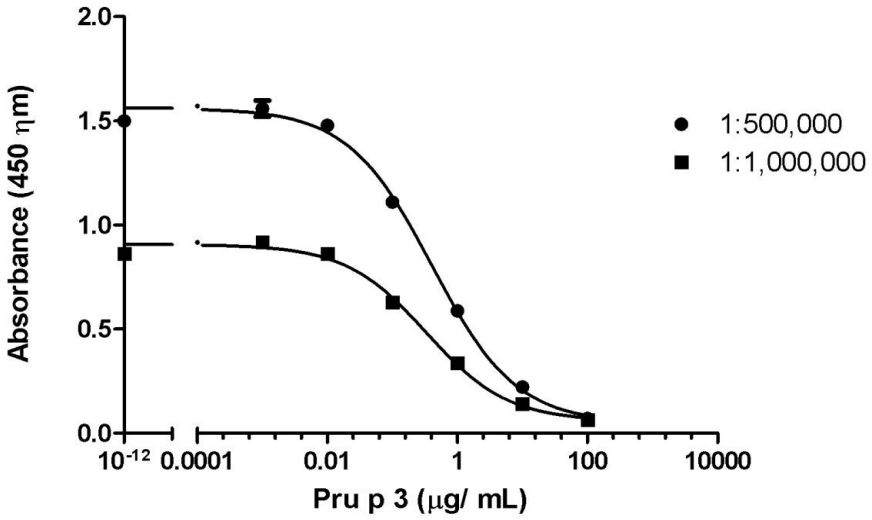


Fig.4.7: Dose response curve of Pru p 3 standard concentrations to two different dilutions (1:500,000 and 1:1,000,000, v: v) of primary antibody (M710). The dilution of secondary antibody was 1:5000 (v: v).

4.3.3.1.1.6 Quantification of Pru p 3 in peach extracts

For quantification of Pru p 3 in peach extracts, ELISA was carried out as described above. The dose response curve for this ELISA is given in Fig. 4.8.

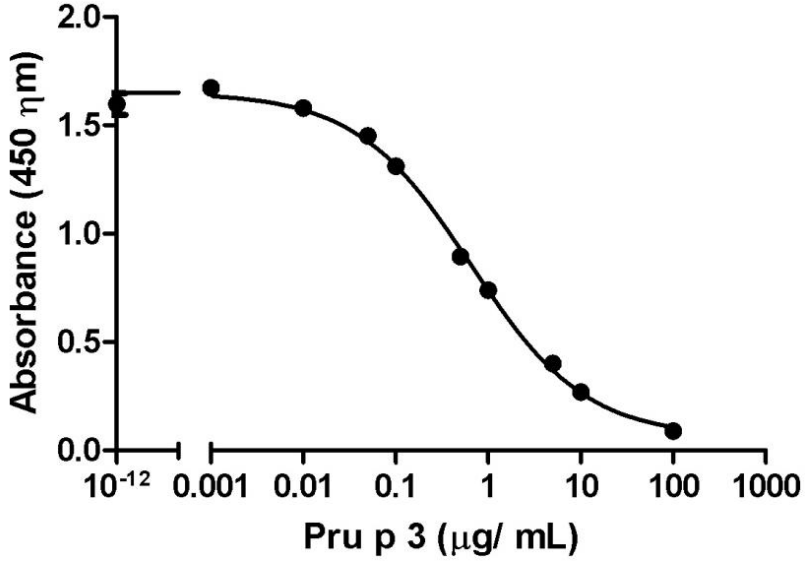


Fig.4.8: ELISA dose response to Pru p 3 with primary antibody dilution at 1:500,000 (v: v) and secondary antibody dilution at 1:5000 (v: v).

The limit of detection in this assay was 10 ng/ mL. The quantity of Pru p 3 determined by this standard dose response in peach peel was found to be 106 ±11 µg/ g of peel while for pulp was 1.7±0.3 µg/ g of pulp. In previous quantifications, Pru p 3 content in whole fruit was quantified as 16.5 µg/ g (Duffort et al., 2002). The present quantification is accordance with this data since peel was separated from the pulp and has already been shown that peel contains at least 10 fold higher content of Pru p 3 than pulp. Our finding is also in accordance with the another finding reporting Pru p 3 content in peel as 132.8 µg/ g of peel and 0.61 µg/ g of pulp (Ahrazem et al., 2007). The Pru p 3 here was found to be 60 fold higher in content in peel than pulp.

4.3.3.1.2 Validation

4.3.3.1.2.1 Spike recovery of Pru p 3 from wheat matrix

For this experiment, the substrate used was TMB being more sensitive than OPD. After calculating the concentration of Pru p 3 in the extracts from wheat spiked with known amount of Pru p 3, it was found that the recovery of Pru p 3 from the wheat matrix is very poor. This is demonstrated by the very low level of Pru p 3 found in the extract (0.25 µg/ mL in the extract obtained with sonication and 0.17 µg/ mL in the extract obtained without sonication) as compared to the quantity spiked (4.26 µg/ mL in each sample). .

4.3.3.1.3 Analysis by ELISA

To investigate the level of Pru p 3 expressed or to confirm the expression of Pru p 3 within the transgenic wheat seeds, ELISA was carried out on the seeds. There were two plasmids pPJ02 with Pru p 3 clone only or pPJ03 with a KDEL at C terminus of synthesized protein to retain it in the endoplasmic reticulum. Out of pPJ02, 34 positive plants were obtained with a positive PCR to Pru p 3 while for pPJ03, there were 11 positive plants with a positive PCR to Pru p 3 gene. There were two plants as negative control for both plasmids. Therefore, there were 47 plants in total yielding 100-200 seeds each. Only eight seeds from a single plant were selected to screen. Pru p 3 levels were measured in the extracts from these seeds and none of the seeds showed Pru p 3 presence (data not shown).

On comparison among the various seeds, the level of Pru p 3 as determined in only a few seeds was found to be extremely low. These levels are found to be in the range where the dose response of ELISA is least sensitive thus representing presence of false positive level of Pru p 3. Since it has already been established that crude extracts of wheat generate background absorbance (data not shown), these false positive values may be a result of the background absorbance.

4.3.3.2 Analysis by immunoblotting

4.3.3.2.1 Dot blot analysis

Ten samples in triplicate each from seeds transformed with PJSA1 and PJSA2 were selected for this analysis. The blot was developed with BCIP/NBT. On comparison between the samples and negative control, it was observed that a background is developed due to the wheat extract. This is also confirmed by comparing positive control (Pru p 3) dilutions prepared either in water alone or in wheat extracts. The same dilutions with wheat extract generated more intense spots than those without wheat extract. These results show that wheat extract alone generates a false positive response. All the samples tested here generated a weak response to the substrate which may be due to the wheat extract alone and is not an indication of the presence of Pru p 3 within the seeds. The data is given in Fig 4.9.

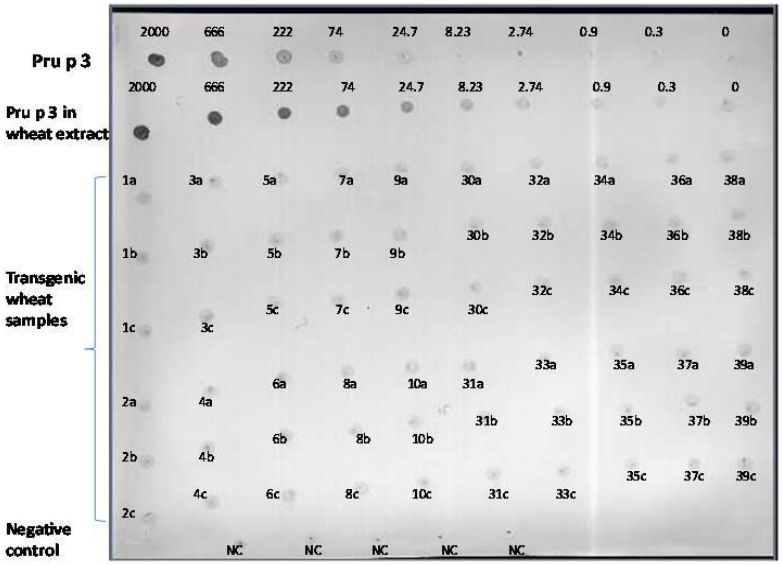


Fig.4.9: Dot blots of transgenic wheat extracts applied directly to the nitrocellulose membrane. First horizontal lane represents various dilutions of purified Pru p 3 in water while second horizontal lane represents the same dilutions of purified Pru p 3 in non-transgenic wheat extract. Bottom lane represents negative control. The sample codes are represented by numeric codes (1,2,3 ---) which represents individual plant and alphabetical codes (a, b, c) which represent individual seeds from the same plant.

4.3.3.3 Analysis by MRM

Multiple reaction monitoring was setup to quantify the levels of Pru p 3 in transgenic seeds. The assay provides the relative quantification of target protein measured with respect to the abundance of a target fragment obtained after over night digestion of purified protein with a suitable enzyme such as trypsin. The digest of the standard is usually diluted at various concentrations and every

concentration is then applied to the mass spectrometer to measure the abundance of target fragment. In an ideal situation, the abundance of a fragment is a function of the quantity of the protein in the sample.

4.3.3.3.1 Development of MRM

For the development of MRM, Pru p 3 was digested under harsh conditions and initially, MALDI – ToF – MS was carried out to determine whether the protein digested fully into its tryptic fragments. This is a prerequisite to monitor the level of expressed Pru p 3 in transgenic seeds. The MALDI – ToF – MS of the digest is given in Fig. 4.10.

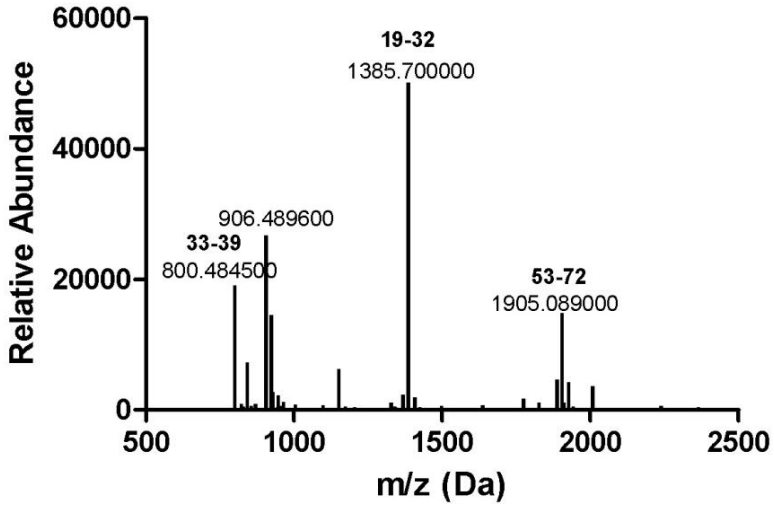


Fig. 4.10: MALDI – ToF – MS of reduced and alkylated Pru p 3 digest with trypsin over night reduced under strong denaturing conditions.

The Pru p 3 digest with trypsin generated only few peptides with the sequences corresponding to the residues 53-72, 19-32 and 33-39 only. There are seven potential cleavage sites of Pru p 3 by trypsin while only five detected here as shown in Fig 4.11.

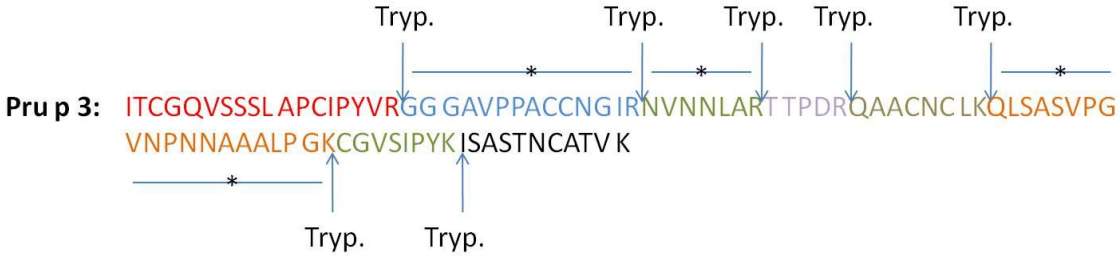


Fig. 4.11: Sequence of Pru p 3 with potential tryptic cleavage sites shown by arrow while those peptides obtained experimentally are shown by "*". Various potential peptides obtained after *in silico* digestion are shown in various colours.

Pru p 3 was not fully digested under the experimental conditions even though none of the cleavable bond is followed by proline (Pro). This explains very stable nature of Pru p 3 being indigestible fully under very strong denaturing conditions i.e heating at boiling for 30 minutes. Again, the digested peptides obtained were not seen completely on MALDI – ToF – MS. For example presence of peptide corresponding to residues 19-32 assures the presence of peptide corresponding to residues 1-18 which was absent in this mode of assay. Similarly a matrix effect is also expected on the digestibility of LTPs. Therefore the digestibility of Pru p 3 within food matrices may or may not follow this pattern of digestibility i.e cleavage at residues 44-45 and 80-81 may occur. Therefore, this method is not suitable for quantification of Pru p 3 within food matrices.

4.3.3.3.2 Digestion conditions

Since the digestibility of Pru p 3 with trypsin did not show fully digestion to yield suitable peptides, chymotrypsin was chosen to develop this method. The digested fragments were again analyzed with MALDI – ToF – MS to investigate digestion profile under these conditions. The MALDI – ToF – MS of the sample is given in Fig. 4.12.

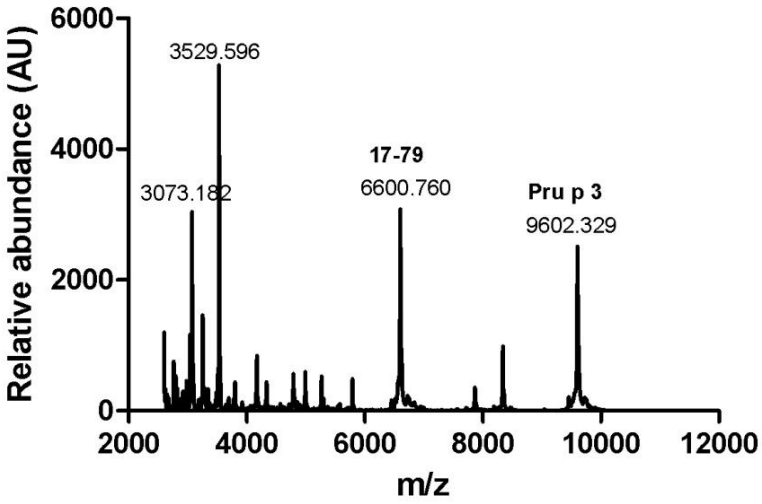


Fig. 4.12: MALDI – ToF – MS of reduced and alkylated Pru p 3 digest with chymotrypsin over night under strong denaturing conditions.

The digestion of Pru p 3 with chymotrypsin under strong denaturing conditions i.e boiling for 30 minutes also generated few peptides along with a high abundance of intact protein which remained un digested. There were several other peaks in the MALDI spectrum which were not identified. Pru p 3 lacks tryptophan (Trp) and phenylalanine (Phe) which are potent digestion sites for

chymotrypsin along with tyrosine (Tyr). Pru p 3 contains only two Tyr residues which were digested in this case generating a high abundance of peptide corresponding to residues 17-79. The generation of this peptide also confirms the presence of residues 80-91 and 1-16 which were not seen on MALDI – ToF – MS. There were other unidentified masses observed which may be a result of autolysis products of enzyme itself. Chymotrypsin hydrolyzes some other peptide bonds also preferably those involving hydrophobic residues but with a much slower rate. These unidentified masses may also correspond to those peptides however; the sequence for these masses could not be identified. Since full digestion of the protein into its fragments is a prerequisite for the technique of MRM, digestion with chymotrypsin did not provide full fragment of Pru p 3 making again, this technique not suitable for quantification of Pru p 3 in food matrices. To observe the matrix effect, peach peel powder was also subjected to proteolysis under the same conditions and MALDI – ToF – MS carried out. The spectrum was devoid of either Pru p 3 or any of its digestion fragments showing the conditions developed are not suitable MRM analysis. The MALDI – ToF – MS of the peach peel digest is shown in Fig. 4.13.

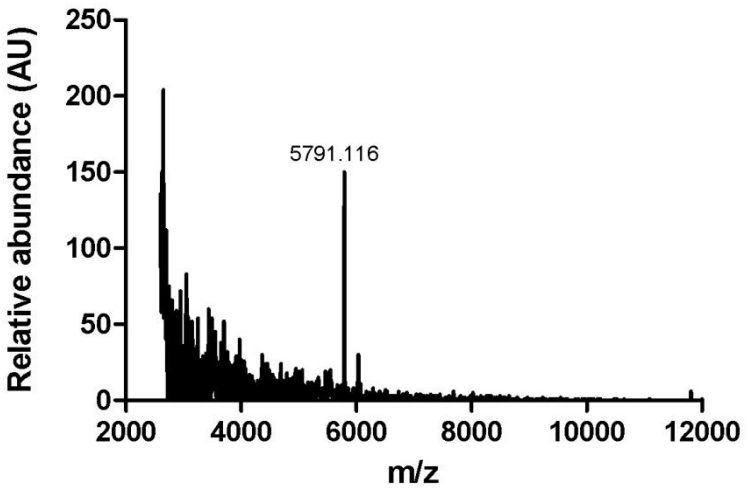


Fig.4.13: MALDI – ToF – MS of peach peel digest with chymotrypsin under reduced and alkylated conditions.

4.4 Discussion and conclusion

The development of clone was successfully achieved as evident by agarose gel electrophoresis of transformants. After removing a fragment of gene from plasmid pLRPT, the target gene cloning Pru p 3 was inserted which was shorter in length than that removed. This resulted in a slightly higher

mobility of the plasmid in the agarose gel medium than the native plasmid. This confirmed that a successful transformation was achieved. The clone was also successfully inserted into wheat seeds as evident by positive bar PCR (Rothamsted Research, Harpenden, UK, result not shown here). However, the expression of Pru p 3 into the wheat seeds could not be achieved as evident by dot blot and ELISA. In dot blot experiment, a false positive blot appeared for all samples which were also seen in the extracts from non-transgenic wheat seeds. This showed that either the transgenic seeds were devoid of any expressed Pru p 3 or a very low level might be present which was not detected under experimental conditions. Therefore, ELISA was established to detect and quantify the levels of Pru p 3 if present in the extracts. Under the experimental conditions, again, a very weak false positive levels detected in some grains which were in the range of ELISA where these levels either may be false positive or may represent the background absorbances. The rest of the seeds showed no detection of Pru p 3. These results explain that either expression of Pru p 3 was not achieved, or the level of expression might be extremely low to detect through conventional procedures or there might very poor extractability of this protein from wheat seeds.

To investigate the extractability of Pru p 3 from wheat seeds, spike recovery experiment was conducted. The spike recovery experiment revealed that wheat matrix has a strong affinity with Pru p 3 since purified Pru p 3 was mixed into the matrix and was unable to recover fully (<6% recovered). The lower extractability may be due to strong affinity of wheat matrix with Pru p 3 or a possible modification of Pru p 3 by the components of wheat resulting in poor solubility in the extracting medium. There might be physical absorption of this protein within the matrix developed after hydrating the wheat flour such as gluten matrix or hydrated starch which entrapped the protein within the matrix and resulted in poor solubility. Therefore, this might be interfering with the extractability of Pru p 3 from the transgenic wheat seed if expressed adequately. Therefore, a careful study is required to investigate the matrix interactions with LTPs. Pru p 3 is well released from the peach peel during the digestion as revealed in our digestion experiments (section 6.3.1.1).

The establishement of multiple reaction monitoring of Pru p 3 also could not be achieved due to the poor digestibility of the protein. MALDI – ToF – MS was used to investigate if Pru p 3 digests fully into its fragments under experimental conditions prior to applying a suitable method for MRM such as orbitrap or ESI-MS. Pru p 3 was not denatured fully under applied conditions which resulted in incomplete digestion. For MRM, Pru p 3 needs to be fully denatured and digested into its fragments so that any of the fragments may be selected for a relative quantification of the digested Pru p 3 into the food samples. The digestibility of purified Pru p 3 was monitored here with MALDI – ToF –

MS and these conclusions were made. This is demonstrated by the fact that MALDI spectrum was devoid of many fragments which were present int the digested mixture such as 1-18 in case of tryptic digestion or 1-16 in case of chymotryptic digestion. For successful monitoring of the levels of Pru p 3 into food samples, a reliable calibration is required with known quantities of digested Pru p 3 and the abundance of the selected fragment as a function of the level of Pru p 3 determined. Chymotryptic digestion was also investigated to determine whether the protein gets digested fully under the digestion conditions. The MALDI – ToF – MS of chymotryptic digest also confirmed that protein was not digestible under the digestion conditions even the digestion was carried out over night.

In conclusion, ELISA was successfully developed to quantify Pru p 3 in various food preparations containing peach or its juice. Either the expression of Pru p 3 into wheat seeds was unsuccessful or the expressed protein remained strongly bound to the wheat matrix and did not come into the solution by extraction buffer which is evident by immunoblotting being absent in any band corresponding to Pru p 3 and ELISA showing most of the seed samples showing no Pru p 3. Therefore, the method developed was unsuccessful for investigation of the effect of food matrix on the digestibility and allergenicity of Pru p 3 but might be of interested to study other less stable allergens. This is mainly because of the problems in expression, extraction and proper identification of digestion fragments of Pru p 3 observed in this case.

5 Effect of ligand binding on digestibility of lipid transfer proteins

5.1. Introduction

Plant lipid transfer proteins (LTPs) are small basic lipid binding proteins which may amongst others have a role in intracellular trafficking of lipid. Along with a motif of eight conserved cysteines, they posses a characteristic α-helical structure conserved in LTPs from various plant species which form a tunnel like cavity. The first three helices are amphiphilic in nature and are parallel to the tunnel while the fourth helix is found at the end of the cavity near the C-terminus to close the end (Yeats and Rose, 2008). The hydrophobic tunnel is able to accommodate a variety of ligands. A more detailed discussion on plant LTPs is given in chapter 1.

The ligand binding behaviour of wheat LTP, Tri a 14 with lipophilic molecules as diverse as fatty acids, prostaglandin and phosphatidyl choline being recognized (Douliez et al., 2002, Guerbette et al., 1999, Pato et al., 2001, Charvolin et al., 1999, Douliez et al., 2000). The tunnel in Tri a 14 can accommodate two ligands (Douliez et al., 2000, Douliez et al., 2002), of these two ligands and covalent adduction of barley LTP (LTP1b) with oxylipin at Asp7 (Bakan et al., 2006) enhanced lipid transfer activity (Bakan et al., 2009) and gastro-duodenal digestibility of the protein (Wijesinha-Bettoni et al., 2010). These results suggest that ligand binding to Tri a 14 may affect its functionality as well as its digestibility profile in the gastrointestinal tract. The effect of ligand binding thus may affect the digestibility of LTPs by stabilizing them against digestion and hence may promote the absorption of immunologically intact form of the epitopes of this class of allergens. There are various food preparations of wheat that include germ and bran portions, which will be rich in Tri a 14 and contain lipophilic compounds that can bind to the protein. Wheat contains 2.2-3.3 % fat, depending upon the variety and season, with the endosperm containing 1.7% and the germ containing 10.9% of lipid (Table 5.1) of the fatty acids found in these different grain fractions the most abundant in linoleic acid.

Table 5.1: Fatty acid composition of wheat lipids.

S. No.	Fatty acids	Whole grain (%)	Endosperm (%)	Bran (%)	Germ (%)
1	Myristic acid (14:0)	0.1	Traces	Traces	Traces
2	Palmitic acid (16:0)	24.5	18.0	18.3	18.5
3	Palmitoleic acid (16:1)	0.8	1.0	0.9	0.7
4	Stearic acid (18:0)	1.0	1.2	1.1	0.4
5	Oleic acid (18:1)	11.5	19.4	20.9	17.3
6	Linoleic acid (18:2)	56.3	56.2	57.7	57.0
7	Linolenic acid (18:3)	3.7	3.1	1.3	5.2
8	Arachidonic acid (20:0)	0.8	Traces	Traces	Traces
9	Others	1.1	1.1	Traces	0.8

Adapted from (Becker, 2008).

In contrast to Tri a 14, the ligand binding properties of Pru p 3 have not been described in detail. The crystal structure of recombinant Pru p 3 showed the tunnel contained two ligands adopted from the culture medium, lauric and heptanoic acid (Pasquato et al., 2006). Binding of saturated fatty acids such as palmitic and stearic acid along with unsaturated fatty acids such as oleic and linoleic acid with Tri a 14 has also been determined and a high affinity was found represented by small Kds ranging submicromolar concentrations (Douliez et al., 2000). Collectively, LTPs from fruits such as peach (Wijesinha-Bettoni et al., 2010, Cavatorta et al., 2010) and grapes (Vassilopoulou et al., 2006) are highly resistant to digestion. It has been shown that tryptic digestibility of BLG was unaffected by binding with retinol, it reduced moderately by binding with palmitic acid (Puyol et al., 1993, Mandalari et al., 2009b). It has not been shown whether Pru p 3 has any effect of ligand binding on its digestibility. Other impacts of ligand binding involve alteration in structure on beer proteins and reduced foaming properties (Cooper et al., 2002) and improvement in DNA binding to response elements (Mochizuki et al., 2006).

Peach is relatively low in lipids as compared to wheat which has a high proportion in germ and bran fractions. Most of the lipids in peach are located in the kernel which is not generally included any food preparation while a small proportion in the waxy layer found in the skin of the fruit. Peach mesocarp contains various classes of lipids such as phospholipids, galactolipids in the form of mono (MGDG) and digalactosyl-diacylglycerol (DGDG), triacylglycerols, diacylglycerols, free sterols and free fatty acids. These lipids may play a role in ligand binding with Pru p 3 during processing and digestion in GI tract. The lipid composition of peach is given in Table 5.2 and 5.3.

Table 5.2: Major lipid classes in peach mesocarp.

Lipid class	Proportion (µmol/ 100 g dry weight)
Phospholipids	442.3
Galactolipids	166.7
Triacylglycerols	7.8
Diacylglycerols	15.7
Free fatty acids	0.5
Free sterols	123.0
Cholesterol	0.5
Campestrol	4.1
Sigmasterol	10.4
Sitosterol	85.0
Total polar lipids	756.3

Adapted from (Zhu and Zhou, 2006) and (Izzo et al., 1995).

Major fatty acids found in all classes of lipids were palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3).

Table 5.3: Fatty acid composition of peach lipids.

S.No	Fatty acids	MGDG (%)	DGDG (%)	TG (%)	DG (%)	FFA (%)	TP (%)
1	Palmitic acid (16:0)	36	57	20	18	33	38
2	Palmitoleic acid (16:1)	3	5	2	2	15	4
3	Stearic acid (18:0)	6	11	2	2	6	6
4	Oleic acid (18:1)	12	13	8	15	22	15
5	Linoleic acid (18:2)	36	5	56	57	25	30
6	Linolenic acid (18:3)	8	5	18	14	0	8

Adapted from (Izzo et al., 1995) and (Zhu and Zhou, 2006).

The aim of this study was to investigate the binding behaviour of Pru p 3 and Tri a 14 in the presence of competitive mixture of ligands. The ligand binding capacity of the purified LTPs was characterized by using a displacement assay. Initially, the binding with a fluorescent lipid cis-parinaric acid was investigated followed by its displacement from LTPs by physiologically relevant ligands based on the methods described (Dansen et al., 1999, Zhenting et al., 2005, Collini et al., 2003, Kane and Bernlohr, 1996, Mogensen et al., 2002, Kennedy et al., 2000, Palmer and Wolf, 1998). The resistance to digestion of LTPs in the presence of ligand has never been shown any where except for adducted form of barley as described above. Therefore, it was included in the study to mimic the behaviour of these allergens into the food matrix during gastro-duodenal digestion. This study thus investigates the effect of ligand binding on the digestibility of LTPs by taking into account the model allergens chosen in this study.

5.2 Methods

5.2.1 Ligand binding

For ligand binding, two methods were employed, one studying the absorbance characteristics of cis-parinaric acid (CPA) while the other studying the fluorescence characteristics as described in section 2.1.6. Beta lactoglobulin was chosen as a test protein and spectrophotmetric assay was performed. Initially, 3 µM CPA in 100 mM phosphate buffer pH 7.5 was scanned between 312-340 nm and absorbance measured. In the next step, 5 µM protein in 100 mM phosphate buffer pH 7.5 mixed with 3 µM CPA was scanned in the same range for the absorbance.

For fluorescence assay, briefly, 5 µM protein in 50 mM phosphate buffer pH 7.5 was titrated with increasing concentration of CPA dissolved in ethanol in a step wise manner keeping the final concentration of ethanol in the mixture below 2% and fluorescence measured. The binding of other

physiologically relevant ligands was investigated using a displacement assay where the LTP - CPA complex is titrated with a displacing ligand dissolved in ethanol in a stepwise manner. This is accompanied by a decrease in fluorescence as the CPA leaves the hydrophobic ligand binding site. LTP was incubated with CPA at or below its K_d (Kane and Bernlohr, 1996, Höfliger and Beck-Sickinger, 2003) which was 1 µM CPA for Pru p 3 and 2 µM CPA for Tri a 14 at pH 7.5 while 0.5 µM CPA for Pru p 3 and 1 µM CPA for Tri a 14 in 150 mM NaCL at pH 2.5. An additional reason for choosing these concentrations of CPA was to keep the fluorescence intensities at a suitable range. The curve fitting of this data was done by rectangular hyperbolic function by using GraphPad Prism which uses Hill's equation (Weiss, 1997).

5.2.2 Ligand loading

The procedure of loading ligand was established by using BLG as a model protein. Linoleic acid was dissolved in 250 mM NaOH at a concentration of 26 mM. 100 µL of this solution was added into 6 mL of simulated gastric fluid containing 5 mg of protein to establish a protein to ligand ratio of 1:5 (w: w) carefully maintaining the pH between 4-7 with 1 M HCl/ NaOH. The protein loaded with linoleic acid was kept in incubator with shaking to stabilize the loaded protein.

5.2.3 Gastroduodenal digestion of LTPs

The gastroduodenal digestion of purified LTPs alone or after loading with linoleic acid, was conducted according to the method described in section 2.2.2. Briefly, purified LTPs were subjected to simulated gastric digestion (150 mM NaCl pH 2.5, Pepsin 1:20 to protein) followed by simulated duodenal digestion (150 mM NaCl pH 6.5, Trypsin, chymotrypsin and protein in 1:4:400 (w: w: w) proportion respectively and 2.5 mM bile salts). The samples were withdrawn at various time points and stored in ice during the experiment while stored at -20°C for longer period. SDS-PAGE was carried out for these samples as described in section 2.1.2.

5.2.4 Mass spectrometry analysis

MALDI – ToF – MS and orbitrap analysis was carried out for the LTP digests under reduced and alkylated form as described in section 2.3. Briefly, 105 µL of protein solution was mixed with 150 µL of digestion buffer (50 mM ammonium bicarbonate) and 15 µL of 100 mM DTT. The solution was vortex mixed and heated at 95°C for 5 minutes. After reduction, alkylation was carried out by adding 30 µL of 100 mM iodoacetamide. The solution was kept in dark for 20 minutes. The sample was diluted to 1 µg/ mL in 0.1 % (v: v) TFA prior to mass spectrometric analysis. The sample is

then either applied in the form of crystalline matrix spots on MALDI – ToF – MS target plates or directly applied to orbitrap mass spectrometer as described in section 2.3.4. The data was analyzed by comparing the *in silico* digestion products of the protein by MMass as desribed in section 2.3.5.2 and comparing the experimental masses obtained.

5.3 Results

5.3.1 Ligand binding studies

5.3.1.1 Binding of cis-parinaric acid to LTPs

The absorbance maximum of CPA alone (321.6 nm) is shifted to 322.8 nm after binding with BLG (Fig. 5.1). This phenomenon is due to the mechanism of transfer of resonance energy between the tryptophan residues of BLG and polyene in CPA (Sklar et al., 1980). BLG contains two tryptophan residues in both isoforms i.e A and B of BLG, Trp^{19} and Trp^{61} out of which Trp^{19} is known to stabilize the bound ligand such as retinol (Katakura et al., 1994). This stabilized form allows Trp^{19} to transfer its resonance energy to the conjugated double bonds of CPA making them at slightly higher energy state requiring slightly less energy to absorb as evident by a very small shift in the absorbance maxima of CPA. However, this phenomenon could not be seen in LTPs because LTPs have no Trp residues while Tyr residues did not show this behaviour.

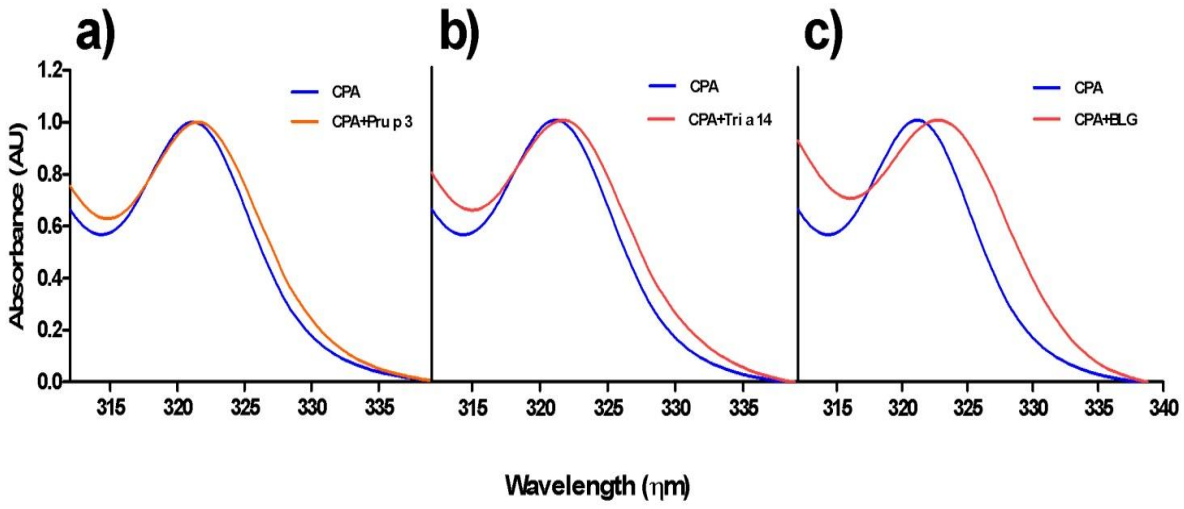


Fig. 5.1: Absorbance spectrum of CPA alone (shown in blue in a, b and c) and mixture of CPA and protein (shown in red). a), b) and c) represent the absorbance spectra of CPA mixed with Pru p 3, Tri a 14 and BLG respectively.

Despite this difference in behaviour, CPA still shows an increase in fluorescence on binding to the LTPs at 420 nm and hence could be used to probe the lipid binding properties of these proteins. Pru p 3 and Tri a 14 are embedded in the food matrix and then are released during digestion. Thus the effect of simulated gastric conditions (150 mM NaCl, pH 2.5) on ligand binding of LTPs was also investigated. Initially, LTPs were titrated with CPA and the fluorescent measured as given in Fig. 5.2.

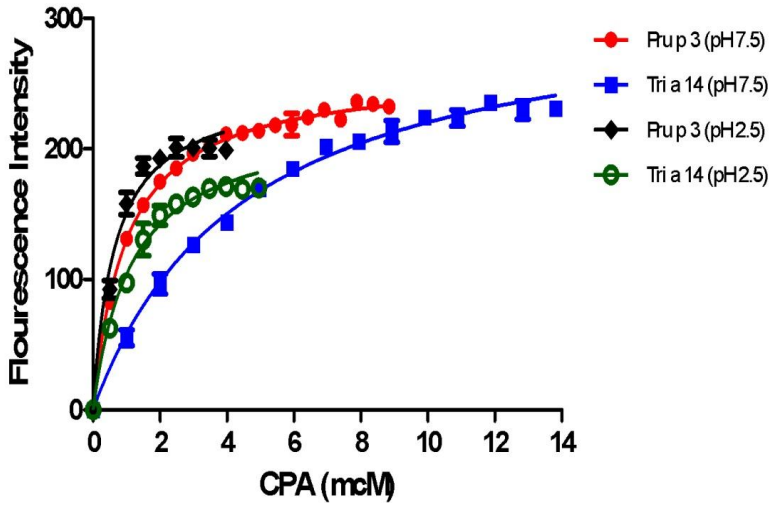


Fig 5.2: Titration of LTPs with increasing concentration of CPA. Fluorescence of CPA was measured at 420 ηm. Point represent the mean of three determinations and error bar represents standard deviation.

The data was collected with the machine which was not functioning well. During the experiment, it was observed that machine had two phases, one when it showed lower sensitivity while after keeping the machine "on" for a few hours, it showed another phase where it was very sensitive to the samples. Keeping the higher sensitivity as standard, all the data was collected in this high sensitive state. Therefore, there is a possibility of the absolute values of K_i and IC_{50}, which might change by using another instrument under the same experiment, the conclusion made by the comparative study of Pru p 3 and Tri a 14 might be the same any where.

The titration curve of Pru p 3 at pH 7.5 was saturable at 5 µM CPA and returned a K_a of 1 ±0.02 µM using Hill's equation. The titration curve of Tri a 14 at pH 7.5 was saturable at 12 µM CPA and returned a K_a of 4.5 ±0.2 µM. The comparison of both LTPs gave evidence that Pru p 3 has much higher affinity with CPA as compared to Tri a 14.

At the low pH and higher ionic strength of gastric conditions, CPA bound slightly more tightly to LTPs as compared to pH 7.5 returning K_a of 0.6 ± 0.1 µM and 1.2 ± 0.1 µM for Pru p 3 and Tri a 14 respectively. There was a greater impact on the saturation condition for the proteins, the concentration of CPA required to saturate CPA biding was at 2.5 and 4 µM to Pru p 3 and Tri a 14 respectively.

5.3.1.2 Comparative binding of other physiological lipids to Pru p 3 and Tri a 14

A displacement assay was then used to study the binding of various ligands. The data was normalized (highest value in each curve was set at 100 while lowest at 0) prior to analyzing the data. An example of the normalized displacement curve for Pru p 3 and Tri a 14 binding palmitic acid at pH 7.5 and phosphatidyl choline at pH 2.5 versus CPA is shown in Fig. 5.3. At pH 2.5, palmitic acid, linoleic acid, 16-OH palmitic acid and 12-OH stearic acid were not sufficiently soluble to be used in ligand binding studies.

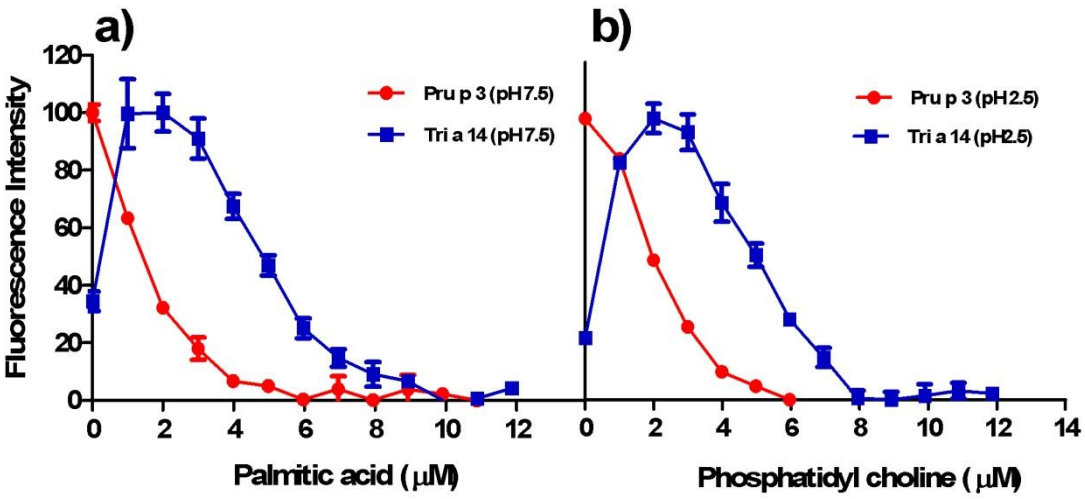


Fig.5.3: CPA displacement assay for LTPs using palmitic acid at pH 7.5 (a) and phosphatidyl choline at pH 2.5 (b) as the competing ligand. Point represent mean of three determinations and the error bar represent standard deviation.

IC_{50} values were derived from these curves and apparent K_is calculated according to Cheng-Prusoff equation (Cheng, 2002) (Table 5.4).

Table 5.4: Comparative IC_{50} and K_i values for a variety of ligands binding to Pru p 3 and Tri a 14, determined using the CPA displacement assay.

Ligand	IC50 (µM)		Ki (µM)		ΔG (Kcal/mol)	
	Pru p 3	*Tri a 14*	*Pru p 3*	*Tri a 14*	*Pru p 3*	*Tri a 14*
Plant lipids						
Palmitic acid (pH 7.5)	1.4±0.1	5.2±0.1	0.7±0.03	3.6±0.1	8.39	7.41
16-OH Palmitic acid (pH 7.5)	4.0±0.1	ND	2.0±0.03	ND	7.77	0
12-OH Stearic acid (pH 7.5)	3.1±0.1	ND	1.5±0.1	ND	7.92	0
Linoleic acid (pH 7.5)	1.6±0.1	7.6±0.03	0.8±0.04	5.3±0.02	8.29	7.19
Digestive biosurfactants						
Phosphatidyl choline (pH 7.5)	2.2±0.03	6.7±0.1	1.1±0.02	4.6±0.1	8.13	7.27
Phosphatidyl choline (pH 2.5)	1.8±0.1	5.3±0.1	1.0±0.1	2.8±0.1	8.17	7.55
Phosphatidyl choline (Vesicle) (pH 7.5)	1.5±0.1	5.6±0.1	0.7±0.04	3.9±0.1	8.35	7.37
Phosphatidyl choline (Vesicle) (pH 2.5)	1.5±0.1	6.8±0.2	0.8±0.1	3.6±0.1	8.29	7.41
Sodium taurocholate + Sodium glycodeoxy cholate (1:1, molar ratio) (pH 7.5)	ND	ND	ND	ND	ND	ND
Lipopolysaccharide (pH 7.5)	ND	ND	ND	ND	ND	ND

*ND: CPA not displaced even at 1:3molar ratio of LTP to ligand.

On comparison between the two groups of ligands, ligands from plants such as palmitic and linoleic displaced CPA more readily and showed higher apparent affinity for LTPs followed by 12-OH stearic acid and 16-OH palmitic acid. Whilst these lipids appeared to bind equally well to Pru p 3, palmitic acid showed higher affinity than linoleic acid for Tri a 14. Despite the binding to Pru p 3, 16-OH palmitic acid and 12-OH stearic acid did not displace CPA from Tri a 14. In the other group of ligands, including gastrointestinal tract surfactants such as bile salts (sodium taurochlate and sodium glycodeoxycholate) and phosphatidyl choline, phosphatidyl choline showed high affinity with LTPs as evident by the relatively low K_i values. Bile salts and lipopolysaccharide did not show any displacement. Interestingly, monomeric PC showed weaker binding to LTPs as compared to its miceller form. The binding energies were calculated by using Gibb's free energy. These results show that binding of all lipid ligands to Tri a 14 was associated with lower free energy than for Pru p 3.

Under gastric conditions (150 mM NaCl, pH 2.5), both vesicular and monomeric PC displaced CPA from Pru p 3, where only monomeric PC showed higher apparent affinity than displacement at pH 7.5. The K_i values for vesicular PC were similar to that at pH 7.5. In the case of Tri a 14, monomeric PC was more effective at displacing CPA compared to pH 7.5 as demonstrated by lower K_i values. The vesicular PC on the other hand displaced CPA weaker than displacement at pH 7.5. Again, Pru p 3 showed higher affinity with ligands under gastric condition as compared to Tri a 14.

5.3.2 Gastroduodenal digestion of purified LTPs liganded with linoleic acid

Linoleic acid was selected as the candidate ligand to investigate its effect on digestibility of LTPs as it is an abundant lipid in peach and wheat and binds with good affinity to both LTPs. Initially, a method for loading the protein with ligand was developed with BLG. Linoleic acid was solubilised in 250 mM NaOH and added to the protein solution slowly to avoid denaturation. The liganded protein was then subjected to gastric digestion which was monitored by SDS-PAGE (Fig. 5.4).

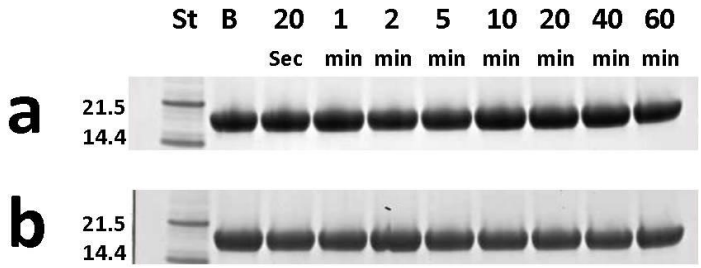


Fig 5.4: SDS-PAGE of the gastric digestion of BLG alone (a) and BLG loaded with linoleic acid (b) performed under reduced conditions. “St” represent standard molecular weight markers lane while “B” represents blank lane in the absence of pepsin.

BLG was found to be stable under gastric conditions and no peptide appeared as digestion product in SDS-PAGE even after 60 minutes of digestion (Fig. 5.4). Linoleic acid did not affect the stability to digestion. After gastric digestion, the protein sample was subjected to duodenal digestion and was monitored by SDS-PAGE (Fig. 5.5).

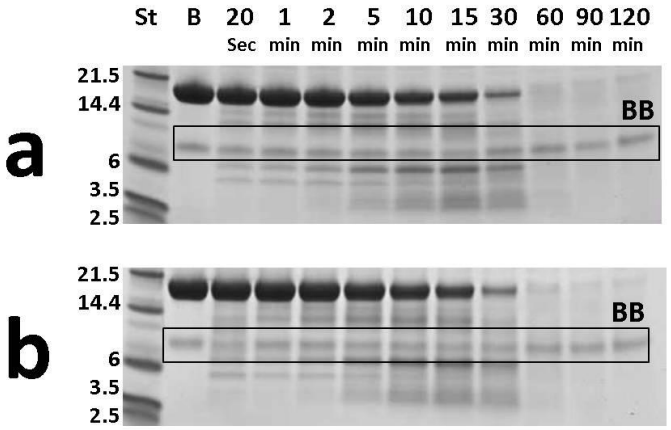


Fig 5.5: SDS-PAGE of duodenal digestion of BLG in the absence (a) and presence (b) of Linoleic acid. BB stands for Bowman birk inhibitor. "St" stands for standard molecular weight marker lane while "B" stands for blank lane without enzymes.

The results showed BLG was digested slowly, with little intact material remaining after 30 minutes digestion. A range of peptides were generated of Mr 3, 3.5, 4.5, 5.3, 7, 10.8, 12.6 and 15.9 kDa with varying abundances as determined by Totallab analysis. The results are in accordance with a previous study (Mandalari et al., 2009a). All these digestion products disappeared after 30 minutes of digestion. No difference was observed in the digestion pattern between the presence and absence of linoleic acid. This approach was then applied to investigate the effect of ligand binding on the digestibility of LTPs.

The CD spectrum of liganded Pru p 3 is identical to the unliganded protein after 60 minutes of gastric digestion showing it was well folded prior to duodenal digestion (Fig. 5.6 e).

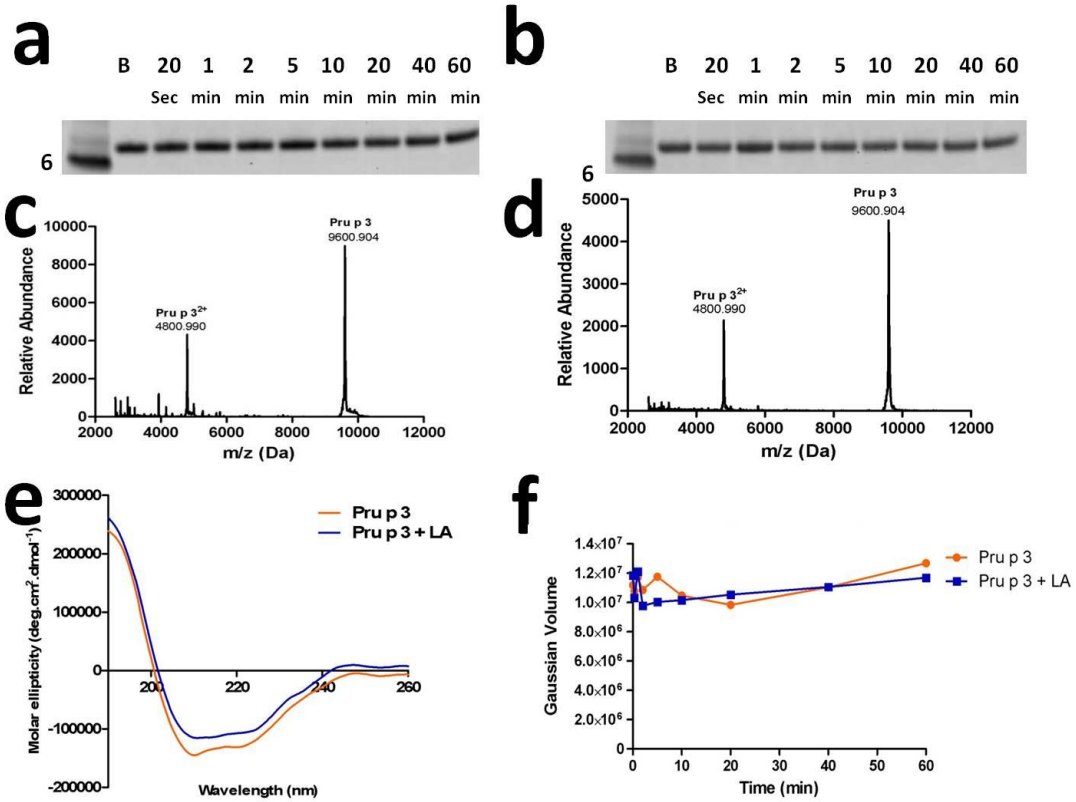


Fig 5.6: Gastric digestion of Pru p 3 alone (a, c) and liganded with linoleic acid (b, d). (a, b) SDS-PAGE analysis of selected time points during digestion under reducing conditions; (c, d) MALDI – ToF – MS of 60 minutes of digestion of the reduced and alkylated protein; (e) CD spectrum of Pru p 3 in the presence and absence of linoleic acid; (f) Densitometric analysis of SDS-PAGE performed in "a" and "b".

Pru p 3 was found to be stable in both liganded and unliganded form during a course of 60 minutes of digestion under gastric conditions as shown in SDS-PAGE and densitometric data (Fig 5.6 a, b and f) with a single intact band of Mr 9 kDa. The samples after 60 minutes of gastric digestion were also analyzed by MALDI – ToF – MS (Fig 5.6 c, d) and circular dichroism spectroscopy (Fig 5.6 e). The MALDI – ToF – MS shows two peaks representing singly charged and double charged intact protein. This had a mass of 9600.9 Da which is identical to the calculated reduced and alkylated mass of Pru p 3 sequence Q9LED1. The CD spectrum of Pru p 3 in the presence and absence of linoleic acid after 60 minutes of digestion showed it remained in a well folded form. After gastric digestion, the protein sample was subjected to duodenal digestion as described in section 2.2.2.

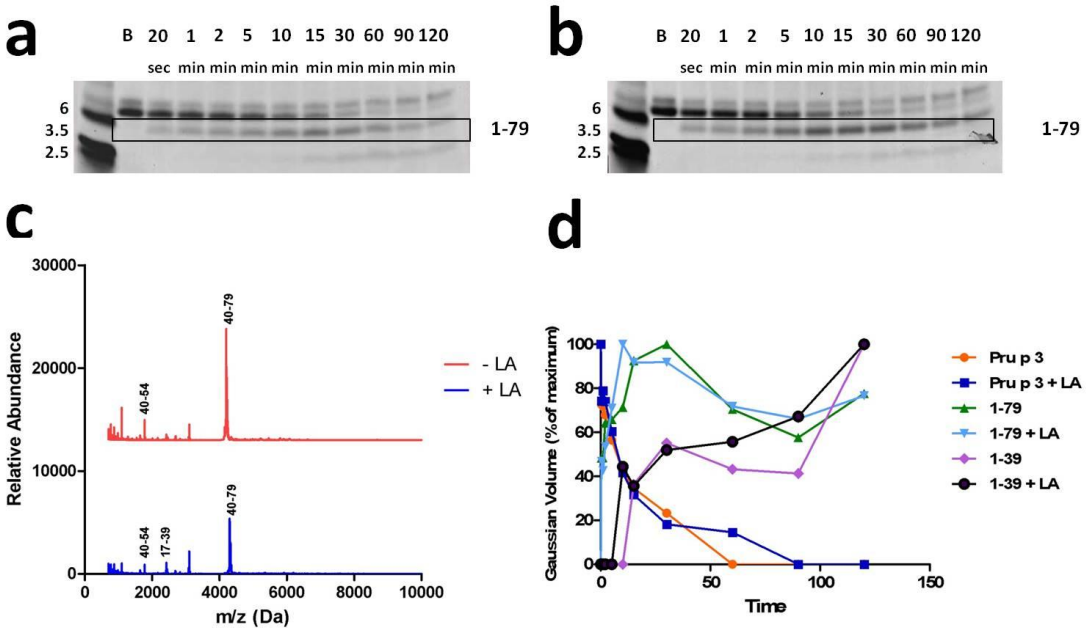


Fig 5.7: Duodenal digestion of Pru p 3 alone (a) and liganded with linoleic acid (b). (a, b) SDS-PAGE analysis of selected time points during digestion under reducing conditions; (c) MALDI – ToF – MS of 120 minutes of digestion of the reduced and alkylated protein; (d) Densitometric analysis of SDS-PAGE performed in "a" and "b". The stable peptide of residues corresponding to residues 1-79 is also highlighted on SDS-PAGE gels.

Pru p 3 was found to be slowly digested under simulated duodenal conditions primarily into peptides of Mr 8334.1 Da and 1279.7 Da which were found to correspond to residues 1-79 and 80-91 (could not be seen here on MALDI – ToF – MS) as determined *in silico* by theoretical digestion of Pru p 3 with enzymes and previous finding (Wijesinha-Bettoni et al., 2010) on the basis of intact masses of the peptides only. The secondary structure pf Pru p 3 is given in Fig. 5.8. The larger peptide 1-79 was further degraded into smaller peptides corresponding to residues 1-39 (4156.1 Da, could not be seen here on MALDI – ToF – MS) and 40-79 (4200.8 Da observed by MALDI – ToF – MS) as determined by mapping the intact masses of the digestion products of Pru p 3 by MMass. Ligand binding did not affect the extent or pattern of proteolysis of Pru p 3. The intensities in the mass spectrum related to the peptides however varied (Fig. 5.7). Another peptide was observed corresponding to residue 40-54 (1774 Da) which is hardly visible by SDS-PAGE either due to its low abundance or poor staining in the gel. Another peptide 17-39 (2424.1 Da) was also observed in liganded Pru p 3 digestion only. The peptides observed here are identical to those already reported for simulated duodenal digestion of Pru p 3 (Wijesinha-Bettoni et al., 2010). These results are also consistent with another study (Cavatorta et al., 2010) but lower in identified peptides mainly

because of the authors used a different proportion of enzymes to protein than those we have already published and followed in the present study.

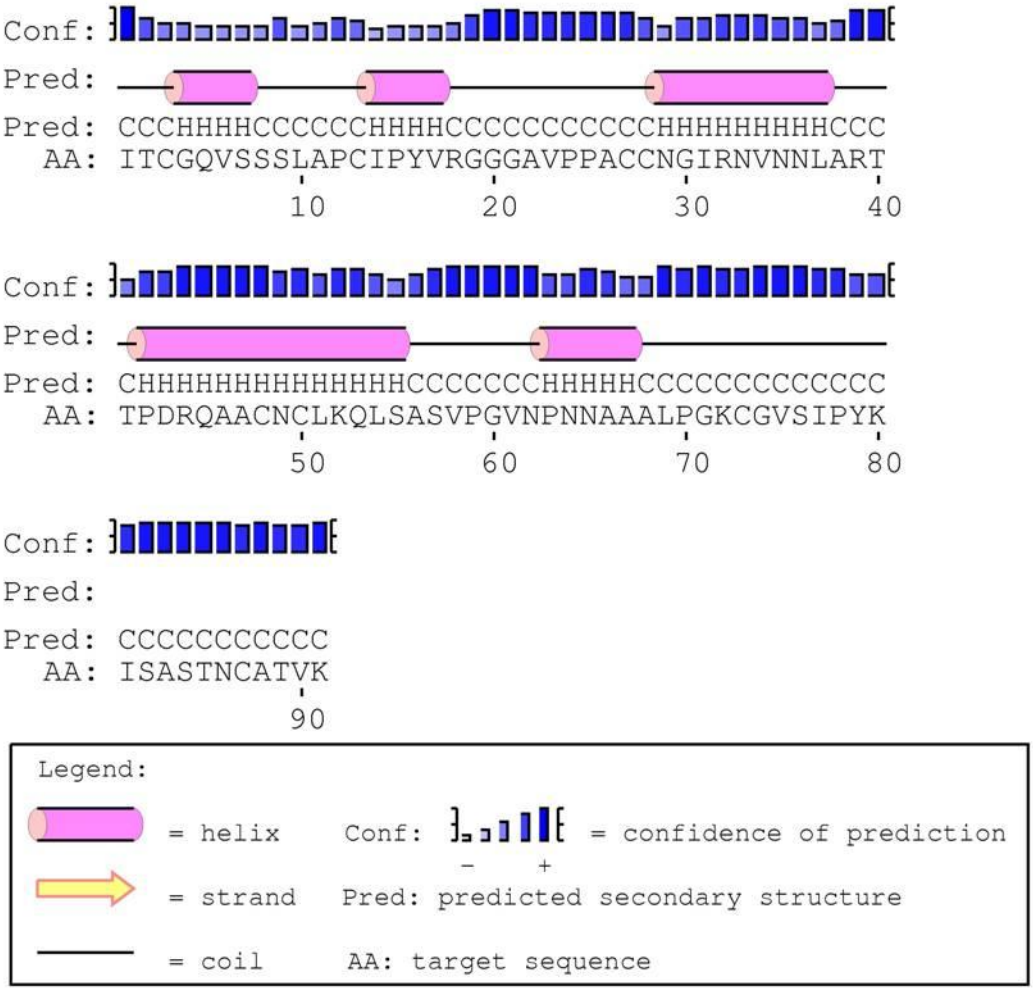


Fig. 5.8: Secondary structure of Pru p 3 showing predicted helices and coils with level of confidence.

The samples obtained here were run on orbitrap mass spectrometer and the data obtained was submitted to MASCOT database search engine to identify smaller peptides of Mr 1-2 kDa. These low abundance peptides, which were not resolved by SDS-PAGE or MALDI – ToF – MS, were identified for both, liganded and unliganded Pru p 3 using a combination of MS – MS on an orbitrap mass spectrometer and mascot database searching. Large peptides were not seen in this analysis. The identified peptides are summarized in Fig. 5.9 and Table 5.5.

Table 5.5: Peptides identified in the duodenal digest of Pru p 3. Residue numbers correspond to those in Q9LED1.

Unliganded Pru p 3						Liganded Pru p 3					
	Orbitrap MS			*MALDI – ToF – MS*			*Orbitrap MS*			*MALDI – ToF – MS*	
Peptide	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**
1-10	1050.502	1050.502	**40-79**	4200.8	4197.1	**1-10**	1050.5	1050.5	**40-79**	4200.8	4197.1
1-16	1751.824	1751.822	**40-54**	1774.9	1775.8	**1-16**	1751.8	1751.8	**40-54**	1774.9	1775.8
17-32	1639.803	1639.804	-	3105.5	-	**17-32**	1639.8	1639.8	**17-39**	2424.1	2422.2
19-32	1384.636	1384.634	-	1097.6	-	**19-32**	1384.6	1384.6	-	1098.5	-
19-37	1938.917	1938.915							-	867.1	-
53-72	1904.011	1904.012				**53-72**	1904.0	1904.0	-	772.3	-
55-72	1662.872	1662.869				**55-72**	1662.9	1662.9			
55-79	2439.227	2439.222									
80-91	1278.66	1278.66				**80-91**	1278.7	1278.7			
81-91	1150.566	1150.565									

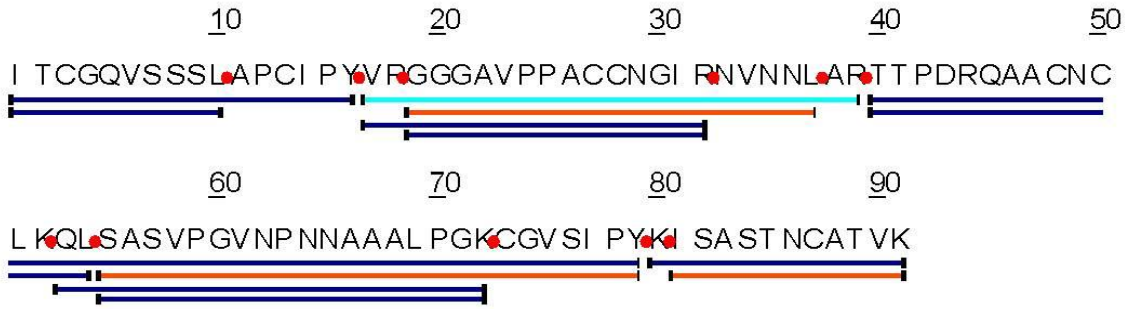


Fig. 5.9: Peptides identified in Pru p 3 due to simulated duodenal digestion. The red dots represent cleavage sites, dark blue bars represent peptides observed in both liganded and unliganded form of Pru p 3, red bars represent peptides appeared in unliganded Pru p 3 only while light blue bars represent peptides appeared in liganded Pru p 3 only.

Like Pru p 3, the spectrum of liganded Tri a 14 was identical to that of the unliganded protein after 60 minutes of gastric digestion, showing it was well folded prior to duodenal digestion (Fig. 5.10 e).

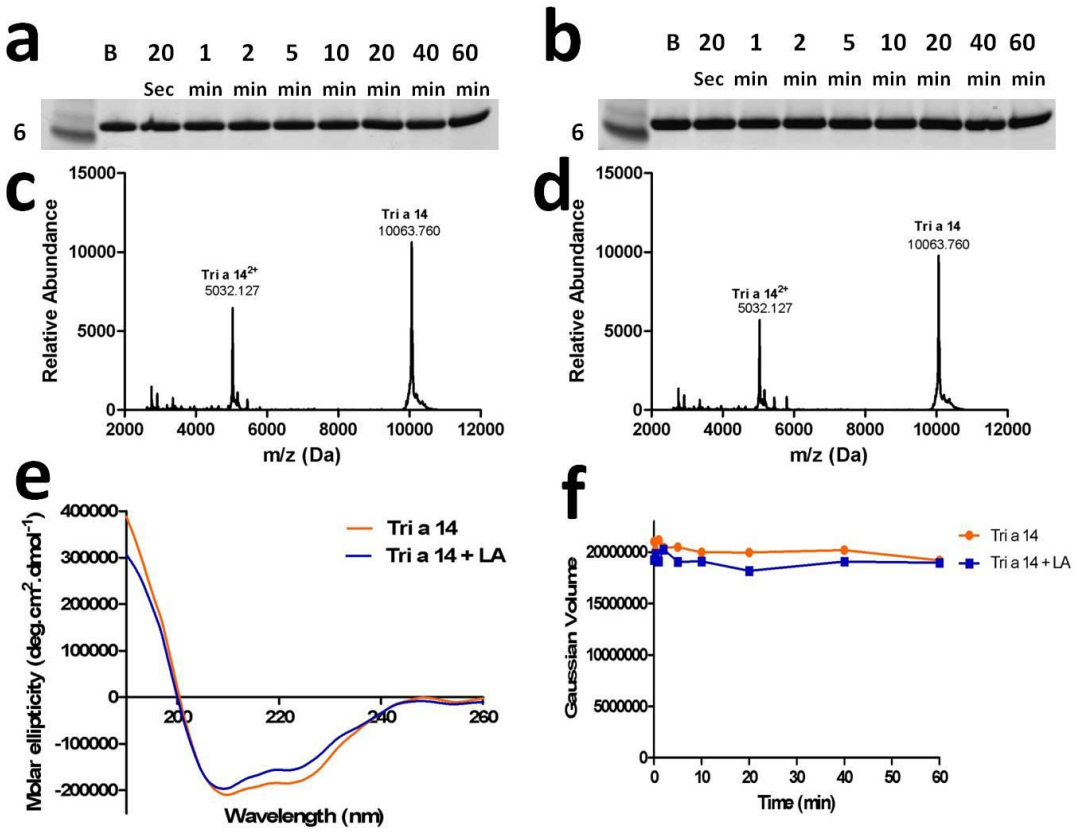


Fig 5.10: Gastric digestion of Tri a 14 alone (a, c) and liganded with linoleic acid (b, d). (a, b) SDS-PAGE analysis of selected time points during digestion under reducing conditions; (c, d) MALDI – ToF – MS of 60 minutes of digestion of the reduced and alkylated protein; (e) CD spectrum of Tri a 14 in the presence and absence of linoleic acid; (f) Densitometric analysis of SDS-PAGE performed in "a" and "b".

Tri a 14 was found to be stable in both liganded and unliganded form during a course of 60 minutes of digestion under gastric conditions as shown in SDS-PAGE and densitometric data (Fig 5.10 a, b and f) with a single intact band of Mr 9 kDa. The samples after 60 minutes of gastric digestion were also analyzed by MALDI – ToF – MS (Fig 5.10 c, d) and circular dichroism spectroscopy (Fig 5.10 e). The MALDI – ToF – MS shows two peaks representing singly charged and double charged intact protein. This had a mass of 10063.7 Da which is identical to the calculated reduced and alkylated mass of Tri a 14 sequence P24296 after removing the signal peptide. The CD spectrum of Tri a 14 in the presence and absence of linoleic acid after 60 minutes of digestion showed it remained in a well folded form. After gastric digestion, the protein sample was subjected to duodenal digestion as described in section 2.2.2.

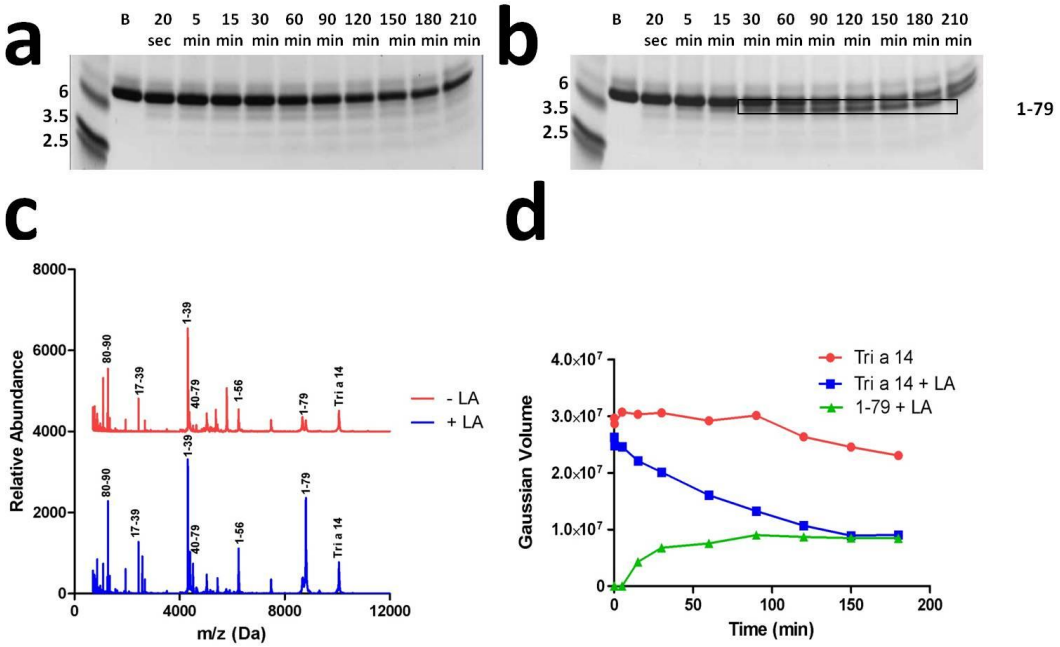


Fig 5.11: Duodenal digestion of Tri a 14 alone (a) and liganded with linoleic acid (b). (a, b) SDS-PAGE analysis of selected time points during digestion under reducing conditions; (c) MALDI – ToF – MS of 120 minutes of digestion of the reduced and alkylated protein; (d) Densitometric analysis of SDS-PAGE performed in "a" and "b". The stable peptide of residues corresponding to residues 1-79 is also highlighted on SDS-PAGE gels.

Unliganded Tri a 14 was more stable to duodenal digestion than Pru p 3, most of the protein (78 % estimated by densitometry) remained intact even after 3 hours of digestion (Fig. 5.11 e). The MALDI – ToF – MS of Tri a 14 after 120 minutes of digestion showed peptides corresponding to residues 1-39, 1-56, 17-39, 40-79, 1-79 and 80-90 (Fig. 5.11 c). The predicted secondary structure of Tri a 14 is given in Fig. 5.12. Some minor peaks in the mass spectrum could not be assigned. The intact Tri a 14 was observed on MALDI – ToF – MS as a minor peak in the mass spectrum (Fig. 5.11 c). The results are similar to the previous study (Palacin et al., 2009) which analyzed the data by SDS-PAGE only.

Loading Tri a 14 with linoleic acid was found to promote duodenal proteolysis and resulted in the formation of stable peptides of Mr 8800.182 Da and 1277.74 Da corresponding to residues 1-79 and 80-90 respectively (Fig. 5.11 d). These peptides were also observed in the unliganded protein but peptide 1-79 was observed at a low abundance in the MALDI – ToF – MS (Fig. 5.10 a, c) and was only present as a faint poly peptide by SDS-PAGE, unlike the liganded protein (Fig. 5.11 b, d). The binding of linoleic acid to the peptide 1-79 however prevented its further degradation and this stable

peptide was observed until the end of the digestion time course. Other peptides resulting from duodenal proteolysis had masses that corresponded to residues 80-90, 40-79, 17-39, 1-56 and 1-39 together with intact Tri a 14. The results are similar to those observed in a previous study showing an increase in digestibility of barley LTP in its adducted form LTP1b, the adduct lying in the binding tunnel of the protein (Palacin et al., 2009, Wijesinha-Bettoni et al., 2010). The identified peptides are presented in Fig. 5.13 and Table 5.6.

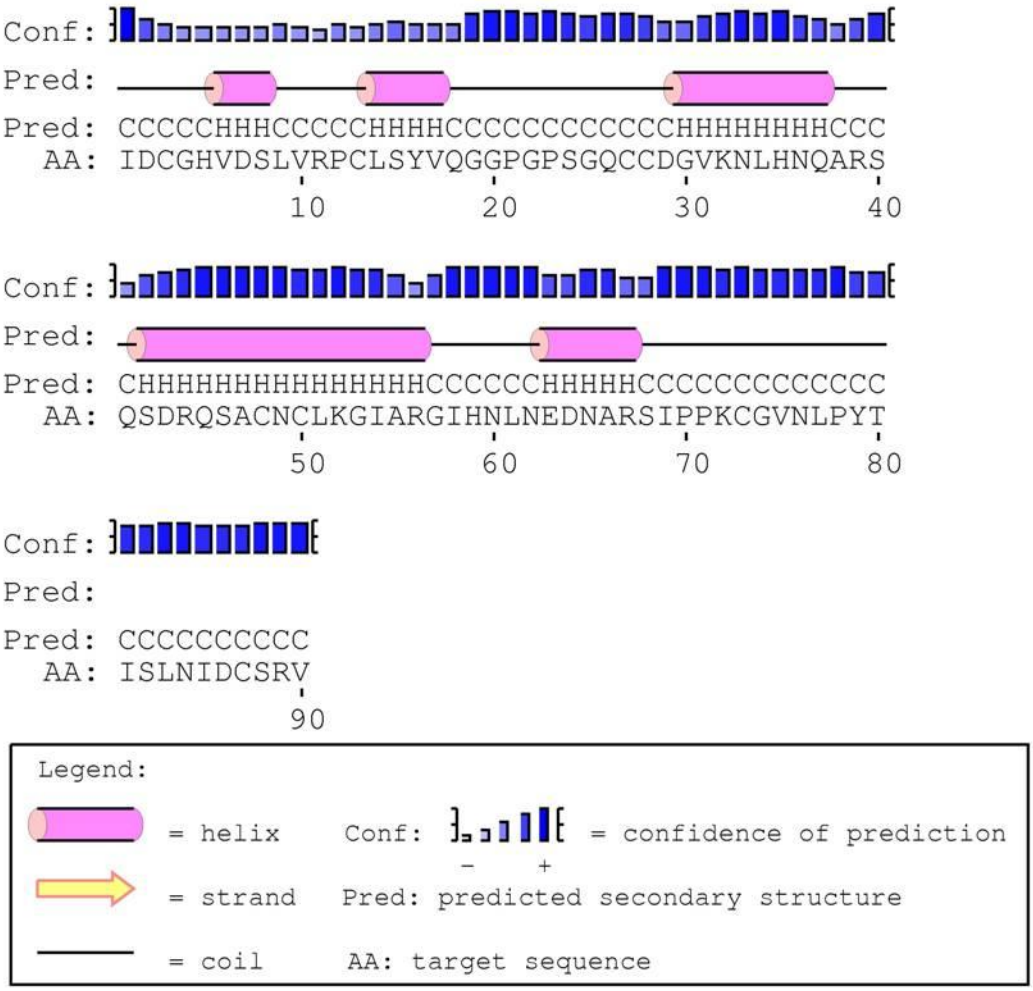


Fig. 5.12: Predicted secondary structure of Tri a 14 showing helices and coils with level of confidence.

Table 5.6: Peptides identified in the duodenal digest of Tri a 14. Residue numbers correspond to those in P24296 excluding the signal peptide.

Unliganded Tri a 14						Liganded Tri a 14					
Orbitrap MS			*MALDI – ToF – MS*			*Orbitrap MS*			*MALDI – ToF – MS*		
Peptide	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**	**Peptide**	**Mr (Observed)**	**Mr (Calculated)**
17-32	1601.6905	1601.6927	**1-90**	10061.350	10057.796	**17-32**	1601.6937	1601.6927	**1-90**	10058.930	10057.796
17-34	1828.8148	1828.8197	**1-79**	8800.182	8799.162	**17-34**	1828.8178	1828.8197	**1-79**	8800.182	8799.162
17-39	2435.1148	2435.1183	**1-67**	7474.997	7473.482	**17-39**	2435.1161	2435.1183	**1-67**	7474.997	7473.482
40-56	1949.9146	1949.9160	**1-56**	6242.674	6239.897	**40-56**	1949.9143	1949.9160	**1-56**	6240.773	6239.897
40-67	3183.4997	3183.5010	**40-79**	4510.413	4510.188	**45-56**	1376.6646	1376.6653	**40-79**	4510.413	4510.1885
57-67	1251.6002	1251.5956	**1-39**	4309.088	4307.991	**57-67**	1251.5925	1251.5956	**1-39**	4307.509	4307.991
68-79	1343.6900	1343.6907	**17-39**	2437.049	2436.125	**68-79**	1343.6892	1343.6907	**17-39**	2437.05	2436.125
80-89	1177.5756	1177.5761	**80-90**	1277.742	1277.652	**80-89**	1177.5761	1177.5761	**80-90**	1277.74	1277.652
80-90	1276.6438	1276.6445	-	1098.537	-	**80-90**	1276.6428	1276.6445	**57-79**	2580.238	2578.283
			-	5035.542	-				**40-56**	1951.931	1950.923
			-	5381.075	-				-	867.108	-
			-	5796.550	-				-	1097.623	-
									-	5033.834	-
									-	5446.584	-

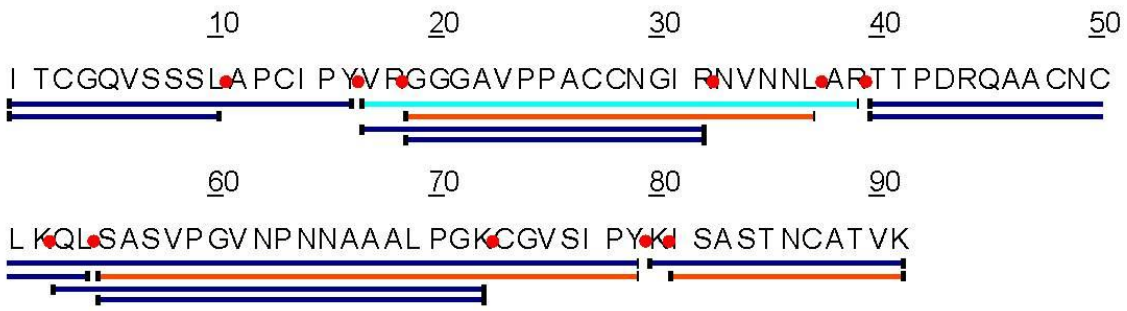


Fig. 5.13: Peptides identified in Tri a 14 due to simulated duodenal digestion. The red dots represent cleavage sites, dark blue bars represent peptides observed in both liganded and unliganded form of Tri a 14, red bars represent peptides appeared in unliganded Tri a 14 only while light blue bars represent peptides appeared in liganded Tri a 14 only.

5.4 Chapter conclusion

Pru p 3 in this study showed higher affinity to all ligands when compared to Tri a 14. The primary sequence similarity between these two proteins is as low as 45% showing that these two proteins are very distinct in primary structure. On comparison between the predicted secondary structures of these two proteins (Fig. 5.8 and 5.11), it is clear that Pru p 3 is slightly more helical (39.56%) than Tri a 14 (38.88%). Further comparisons revealed that both of the proteins have three large and two small helices called helices 1-5 from N-terminus. Helices 2 and 5 are absolutely conserved both in position and length while differences were found between the pairs of helices of these two proteins. The first helix of Pru p 3 is closer to N-terminus and is larger in length than that of Tri a 14. Similarly, the third helix in Pru p 3 is again, closer to N-terminus than Tri a 14 and larger in length. The fourth helix is equidistant from N-terminus in both proteins but this helix is larger in Tri a 14 than Pru p 3.

Besides the sequence and secondary structure differences between these two proteins, the higher affinity of Pru p 3 to ligands may also be due to other differences. For example, this difference may be due to the fact that Tri a 14 is more plastic (Charvolin et al., 1999) and has bigger cavity than Pru p 3 (Pasquato et al., 2006). The bigger cavity of Tri a 14 allows not only more than one ligand but the distance between the ligand and the biding sites within the cavity is higher making it weakly interacted as compared to Pru p 3 with smaller cavity and hence, smaller distance. The difference in affinity to ligands may also be due to the reason that Pru p 3 has eight basic and one acidic residue while Tri a 14 has nine basic and seven acidic residues. Therefore, the overall surface charge of Tri a 14 is higher than Pru p 3 making it slightly unfavourable for hydrophobic interaction than Pru p 3. Finally, the hydrophobicity of Pru p 3 (0.082) is much higher than Tri a 14 (-0.400) as determined

by GRand AVerage of hydrophobicitY (GRAVY, Expassy Tools). This makes Pru p 3 more favourable for hydrophobic interaction than Tri a 14. The ligand binding at lower pH was found to be higher than neutral pH. This result may be due to the fact that at low pH, the solution contained 150 mM NaCl to simulate the gastric fluid which in turn increases the hydrophobicity within the cavity of protein and hence, improves ligand binding (Bonomo et al., 2006).

The digestibility of LTPs has been well studied in both digestive compartments i.e gastric and duodenal (Vassilopoulou et al., 2006, Cavatorta et al., 2010, Wijesinha-Bettoni et al., 2010, Palacin et al., 2009). The effect of ligand binding on the digestibility has been shown by decreasing the digestibility in many cases such as BLG (Mandalari et al., 2009b). In our study, Pru p 3 showed no change in its digestibility pattern through SDS-PAGE while showed the recognition of additional peptides such as 17-39 by MALDI – ToF – MS and disappearance of some peptides as determined by orbitrap MS (Fig. 5.9, Table 5.5). This might be due to the fact that ligand binding does not affect the digestion sites in Pru p 3 (Data not shown, paper in preparation). In case of the digestibility of Tri a 14 in the presence of ligand, it is in contrast to those findings to Pru p 3 and BLG (Puyol et al., 1993, Mandalari et al., 2009b) and the original hypothesis drawn which was a decrease in the digestibility. Here, we report that after binding with ligand, Tri a 14 is more digestible. This may be because of LTPs being small in volume and highly affected in their peptide mobilities by ligand binding such as in case of barley LTP (Wijesinha-Bettoni et al., 2010, Wijesinha-Bettoni et al., 2007). This higher mobility of the peptide residues increased the digestibility which may in turn, affects the epitope recognition in wheat allergic patients. This is because of the location of residues digested under simulated conditions being highly affected by lipid binding (unpublished data, paper in preparation). It was clear from simulation studies that ligand binding to Tri a 14 increased the mobility of the residue 79 which was found to be the initial cleavage point in this protein by chymotrypsin. Therefore, this study confirms that LTPs may become more digestible in their native food matrices by adopting ligands from complex food components or may remain unaffected. In conclusion, ligand binding to LTPs may have various effects depending upon the 3-D structure of LTPs which is over all conserved in nature but differences are found among residues of the members of this class of protein. This make them versatile in their digestibility patterns as demonstrated here by taking into account of Pru p 3 and Tri a 14.

6 Effect of processing and food matrix on digestibility of lipid transfer proteins

6.1 Introduction

Heat treatment may alter the protein SDS-PAGE profile of foods by losing some of the fractions such as in tuna and salmon. The unheated protein extract showed various bands on gel out of which, some disappeared after heating the fish (Taylor et al., 2004). A common way of processing foods is to apply heat and ranges from gentle heating such as pasteurization of milk at 71.7°C for more than 17 seconds (Roth-Walter et al., 2008) to more severe processing such as canning of carrots at 121°C for 15 minutes at 15 Psi (Mishra and Sinha, 2011). Heating causes proteins to denature. At 55-70°C, many animal proteins tertiary structure is lost while reversible unfolding of secondary structure occurs. At temperatures 70-80°C, cleavage of disulphide bonds occur while aggregates are formed in the range of temperature 90-100°C. Chemical modifications occur in the range of 100-125°C or even above (Wal, 2003). The chemical modifications include scrambling of disulphide bonds and random hydrophobic interactions resulting in non-specifically interacting proteins leading to dimmers and aggregated proteins (Davis et al., 2001). The effect of heat treatment to LTPs has been described and was found that LTPs from fruits such as from cherry (Scheurer et al., 2004) and peach (Gaier et al., 2008) are denatured on heating until 100 °C but show resistance to heat in case of cereals such as wheat (Palacin et al., 2009) and barley (Matejkov et al., 2009). The effect of heat treatment to LTPs on their digestibility has not been studied yet. This study first time reports the effect of thermal treatment of LTPs on their digestibility.

The effect of food matrix is a big challenge to study. Studies are in progress to investigate the effect of food matrix on allergenicity and digestibility. One component from fruits, pectin was found to decrease the digestibility of allergens such as thaumatin like proteins in kiwi, banana, apple and celery by pepsin (Polovic et al., 2007). The solubility of protein from legumes was investigated and was found that solubility of proteins in raw form is minimum at pH 4 while above and below this pH, solubility increases. In case of cooked form, the solubility is achieved at or above pH 10 (Carbonaro et al., 1997). The effect of cooking on legumes protein digestibility varies among legumes e.g increases in chickpea and dry beans while decreases in faba beans (Carbonaro et al., 1997). The effect of *in vitro* digestibility of almond proteins as determined by protein retained in the insoluble material post digestion explained that only < 50% of the total proteins disappeared after digestion (Mandalari et al., 2008). Various processing such as dehulling, germination and extrusion improved the protein content and digestibility of the legumes such as faba and kidney beans (Alonso et al., 2000). The effect of boiling and pressure cooking in some varieties of rice either did

not change or reduced the protein content however, protein digestibility of all the rice varieties improved (Sagum and Arcot, 2000). In case of wheat products such as dough, bread crumb and crust, the pepsinolysis digested the high molecular weight proteins in dough and crumb quickly while in crust, they became immobile on SDS-PAGE gel and remained at top of the gel even after the end of digestion while in the duodenal phase of digestion, the proteins with low molecular weight remained undigested (Pasini et al., 2001, Simonato et al., 2001).

There are no general rules to follow for the effect of thermal treatments on allergenicity. The effect of heat treatment to allergens may alter their allergenicity by creating either new epitopes such as in case of fish (Prausnitz and Kustner, 1921) and BLG (Williams et al., 1998) or reducing the allergenic activity such as in case of egg (Urisu et al., 1997). The extent of allergenicity however mainly depends upon the temperature, duration and intrinsic properties of allergen. Advanced glycation end products together with oxidized lipids, oxidation of proteins, disulphide scrambling and deamination of asparagine also occurs during food processing. Polyphenol interaction with proteins is also a common reaction to food proteins. These modified proteins may play a significant role in allergic reactions being important B-cell epitopes (Davis et al., 2001). In some cases, processing does not alter the immune response such as heating, boiling and extrusion of lupine (Alvarez-Alvarez et al., 2005). The food matrix effect is also important in investigating the IgE recognition. In case of Bet v 1 homologues of apple and celery, the purified protein lost its immunogenicity while retained in crude or partially purified extracts (Vieths et al., 1998).

The present study takes into account of model thermal processing of purified proteins and food matrices to mimic canning process (Brenna et al., 2000) and its impact on protein digestibility.

6.2 Methods

6.2.1 Model thermal processing

Model thermal processing was carried out to investigate the effect of processing on the structural stability to digestion of these lipid transfer proteins. Peach processing conditions i.e heating at 121 °C for 15 minutes was selected as a model for purified proteins and heat treatment to peaches while model bread was made to study the food processing of wheat.

6.2.1.1 Thermal processing of purified proteins

To mimic the commercial processing of peaches, LTPs were dissolved either in water or in 30% sucrose at 0.2 mg/ mL and sealed in a screw capped tube. The tube was heated in a hot block previously heated at either 90°C or 121°C for 15 minutes. Finally, the tube was removed from the

hot block and cooled at room temperature. For digestion experiment, BLG or LTPs were dissolved in water at 10 or 5 mg/ mL respectively and sealed in a capped tube. The tube was heated in the hot plate at 121°C as described above and cooled to room temperature.

6.2.1.2 Thermal processing of peaches

Thermal processing of peaches was carried out by covering fresh peaches (obtained from a local market in Norwich, UK) either in de-ionized water or in 30% sucrose solution in a large glass beaker. The beaker was covered with aluminium foil and heated in an autoclave at 121°C and 15 Psi for 15 minutes. After heating, the peaches were cooled down to room temperature and the peel was separated from the pulp by using a spatula. The peel and pulp were subjected to simulated gastric and duodenal digestion as described in section 2.2.4 with a difference that cooked peaches were not chewed by the mincer but crushed finely with spatula before subjecting to digestion.

6.2.1.3 Thermal processing of wheat

To investigate the digestibility of Tri a 14 in its native matrix, wheat bread was prepared and subjected to gastroduodenal digestion. A rapid whole wheat bread was made according to (Shapter, 2001). All the ingredients were obtained from a local market in Norwich. Briefly, 375 g whole wheat flour was mixed with 210 mL water, 22 mL sunflower oil, 15 g skimmed milk powder, 7.5 g table salt, 15 g table sugar and 5 g dried yeast. The ingredients were mixed and cooked to final bread in a moulinex home bread maker (Group SEB France, Ecully Sedex, France) according to manufacturers instructions.

6.2.2 Circular dichroism analysis

Circular dichroism analysis of the heat treated purified proteins was carried out as described in section 2.1.6. Briefly, the CD spectrum of 0.2 mg/ mL heat treated protein solution either in water or in 30% sucrose was measured from 270 – 190 nm with J-710 spectropolarimeter (Jasco Corp. Tokyo, Japan) against the same buffer by using a 0.1 mm path length quartz cell. Spectra represents the average of four accumulations collected at 20 nm/ min with a 4 sec. time constant, a 0.5 nm resolution and sensitivity ±100 mdeg.

6.2.3 Simulated gastroduodenal digestion

6.2.3.1 Digestion of heat treated proteins

BLG was chosen as test protein for this assay. One millilitre of the heat treated protein solution was mixed with 8 mL of SGF and the pH returned to 2.5 using 1 M HCl/ NaOH. Finally, the volume

was made up to 9.9 mL by SGF and digestion carried out as described in section 2.2.3. SDS-PAGE and MALDI – ToF – MS of the samples were carried out as described in section 2.1.2 and 2.3.

6.2.3.2 Digestion of food matrices

Native peach peel and wheat flour fractions (commercial whole wheat flour, white flour, wheat bran and laboratory milled whole wheat flour from cv Cadenza), heat treated peaches or bread prepared were subjected to simulated gastroduodenal digestion as described in section 2.2.4. SDS-PAGE, in gel trypsin digestion and mass spectrometry was carried out as described in section 2.1.2 and 2.3.

6.2.4 SDS-PAGE

SDS-PAGE of the digests of food matrices was carried out as described in section 2.1.2. Briefly, at the end of digestion, the digested material was centrifuged at 22100 x g to separate the supernatant and pellet. Supernatant was applied to SDS-PAGE as described earlier. In case of pellet, an extraction buffer was prepared by diluting 4xLDS sample buffer into water to a final concentration of 1xLDS sample buffer and 100 mM DTT. This extraction buffer was applied as 8:1 (v: w) to pellet followed by vortexing and heating at 70°C for 10 minutes. Finally, this extract was centrifuged at 22100 x g for 10 minutes and the supernatant applied directly to SDS-PAGE gel.

6.2.5 Immunoblotting

Immunoblotting was carried out for the digestion products of peaches only. The samples were prepared according to SDS-PAGE sample preparation and the method was followed as described in section 2.4.4.

6.2.6 Mass spectrometry

MALDI – ToF – MS analysis was carried out for the LTP digests without reduction and alkylation. The sample was diluted to 1 µg/ mL in 0.1 % (v: v) TFA prior to mass spectrometric analysis. The sample is then applied in the form of crystalline matrix spots on MALDI – ToF – MS target plates. In gel trypsin digestion was carried out as described in section 2.3.3.

6.3 Results

6.3.1 Model thermal processing of LTPs

The CD spectrum of Pru p 3 before heat treatment is predominantly helical as described in literature and is well folded with a helical content of 62% (Fig. 6.1). When the protein was heated to 90°C and cooled, the spectrum remained the same in its well native folded form. On comparing structural components, it was observed that a slight increase in helical structure (75%) occurred along with

slight reduction in disordered random structure. In case of heating at 121°C, the predominant helicity of the structure disappeared and the protein's native secondary structure was fully lost. This was affirmed by the fact that helicity of this denatured protein dropped to 13.4% with a considerable increase in β-sheet, turns and random disordered structure (Table 6.1).

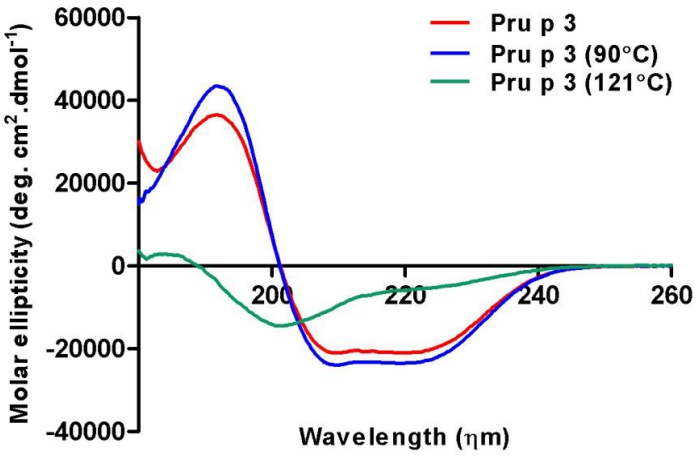


Fig. 6.1: Circular dichroism spectroscopy of purified Pru p 3 in water measured at 25°C after giving various heat treatments as shown in figure legend.

Table 6.1: Structural components of Pru p 3 as determined by CD-Pro.

	CDSSTR					ContinLL			
	Helix	*Sheet*	*Turn*	*Random*		*Helix*	*Sheet*	*Turn*	*Random*
Pru p 3									
IBasis 3	0.67	0.09	0.08	0.16	**IBasis 3**	0.60	0.02	0.16	0.22
IBasis 4	0.62	0.08	0.12	0.18	**IBasis 4**	0.59	0.03	0.16	0.22
IBasis 6	0.65	0.06	0.08	0.21	**IBasis 6**	0.58	0.04	0.15	0.23
IBasis 7	0.65	0.07	0.11	0.17	**IBasis 7**	0.59	0.03	0.16	0.23
Pru p 3 (90°C)									
IBasis 3	0.81	0.05	0.05	0.10	**IBasis 3**	0.66	0.03	0.13	0.18
IBasis 4	0.68	0.07	0.10	0.14	**IBasis 4**	0.64	0.03	0.16	0.16
IBasis 6	0.79	0.03	0.08	0.10	**IBasis 6**	0.66	0.03	0.13	0.18
IBasis 7	0.75	0.04	0.07	0.14	**IBasis 7**	0.65	0.30	0.15	0.17
Pru p 3 (121°C)									
IBasis 3	0.19	0.25	0.24	0.33	**IBasis 3**	0.20	0.19	0.24	0.37
IBasis 4	0.14	0.26	0.26	0.34	**IBasis 4**	0.13	0.19	0.26	0.42
IBasis 6	0.10	0.13	0.11	0.66	**IBasis 6**	0.80	0.14	0.13	0.65
IBasis 7	0.09	0.12	0.11	0.68	**IBasis 7**	0.15	0.10	0.12	0.63

The effect of heat treatment in the presence of sucrose could not provide structural information on the protein as sucrose itself appeared as a predominant positive peak in the CD spectrum and masked the spectrum of protein (data not shown).

The heat treatment at 90°C did not show a major change in the intact mass of the protein which remained predominantly a single peak representing intact Pru p 3 along with some minor peaks representing additional masses of 18 and 36 Da which may be due to addition of water molecules (Fig. 6.2 a). Heat treatment of Pru p 3 at 121°C generated additional peaks corresponding to

addition of 23 Da and loss of 32 and 64 Da. This may be due to addition of sodium from the buffer and removal of sulphur from cysteine residues of the protein as already described for apple LTP (Sancho et al., 2005).

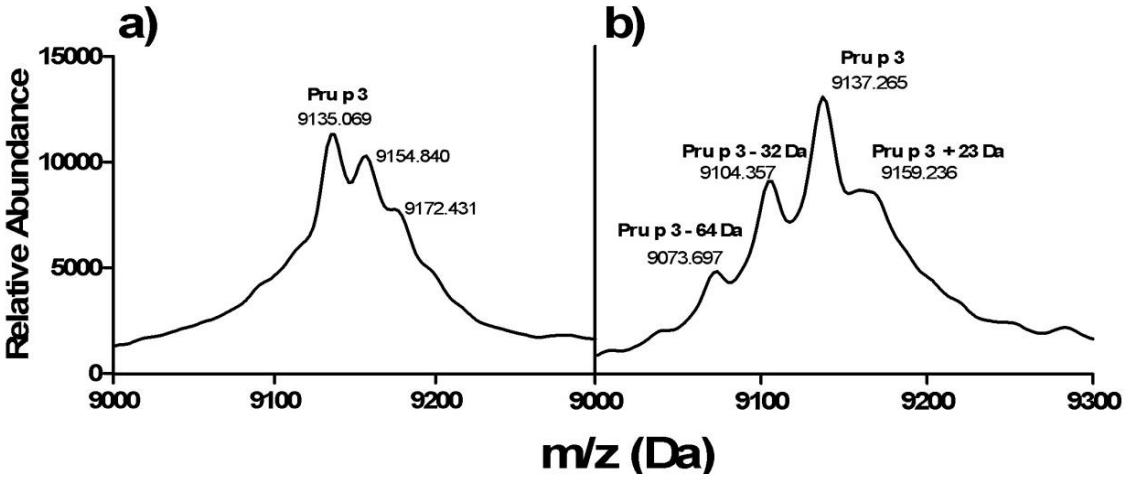


Fig. 6.2: MALDI – ToF – MS of Pru p 3 heated at 90°C (a) and 121°C (b).

The secondary structure of native Tri a 14 was well folded and is primarily a helical structure as described in literature with a helical content of around 55% (Fig. 6.3). The effect of heating at 90°C did not show any major change in the protein secondary structure and hence, it remained the same in well folded form. However, on calculating the helical content when heated at 90°C, the helicity increased to around 60% with a decrease in disordered random structure. In case of heating at 121°C, the protein secondary structure was partially denatured but still retaining major helical form. The helicity dropped to 42% with an increase in β-sheet, turns and disordered random structure. The structural components of Tri a 14 are given in Table 6.2.

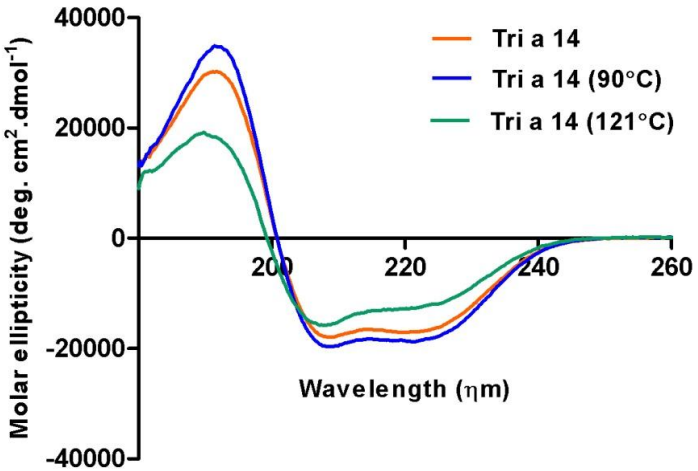


Fig. 6.3: Circular dichroism spectroscopy of purified Tri a 14 in water measured at 25°C after giving various heat treatments as shown in figure legend.

Table 6.2: Structural components of Tri a 14 as determined by CD-Pro.

	CDSSTR					ContinLL			
	Helix	*Sheet*	*Turn*	*Random*		*Helix*	*Sheet*	*Turn*	*Random*
Tri a 14									
IBasis 3	0.58	0.07	0.14	0.21	**IBasis 3**	0.53	0.05	0.18	0.23
IBasis 4	0.56	0.07	0.14	0.23	**IBasis 4**	0.52	0.05	0.19	0.24
IBasis 6	0.57	0.09	0.12	0.23	**IBasis 6**	0.51	0.06	0.17	0.26
IBasis 7	0.57	0.06	0.10	0.27	**IBasis 7**	0.52	0.04	0.17	0.27
Tri a 14 (90°C)									
IBasis 3	0.64	0.06	0.12	0.18	**IBasis 3**	0.60	0.03	0.17	0.20
IBasis 4	0.61	0.05	0.12	0.21	**IBasis 4**	0.58	0.04	0.18	0.20
IBasis 6	0.63	0.05	0.12	0.20	**IBasis 6**	0.57	0.04	0.16	0.23
IBasis 7	0.64	0.05	0.13	0.19	**IBasis 7**	0.58	0.03	0.16	0.23
Tri a 14 (121°C)									
IBasis 3	0.45	0.13	0.19	0.28	**IBasis 3**	0.41	0.08	0.22	0.28
IBasis 4	0.41	0.16	0.18	0.27	**IBasis 4**	0.41	0.09	0.20	0.30
IBasis 6	0.43	0.09	0.15	0.33	**IBasis 6**	0.39	0.07	0.22	0.32
IBasis 7	0.43	0.09	0.15	0.32	**IBasis 7**	0.40	0.07	0.18	0.34

The effect of heat treatment on Tri a 14 in the presence of sucrose did not provide structural information on the protein as sucrose appeared as the predominant peak in the CD spectrum and masked the spectrum of the protein (data not shown).

Heat treatment of Tri a 14 at 90°C produced additional peaks corresponding to addition of 96 Da which might be due to addition of three molecules of oxygen as a result of oxidation (Sancho et al., 2005) and an unidentified peak with molecular weight 9764 Da (Fig. 6.4 a). However, the major peak still remained to be the native intact Tri a 14. On heating at 121 °C, Tri a 14 appeared as native Tri a 14 in the form of a single peak with the right molecular mass with additional peaks corresponding to loss of 18 Da and 32 Da. This may be due to a possible dehydration and loss of one sulphur in the form of SO_2 (Sancho et al., 2005).

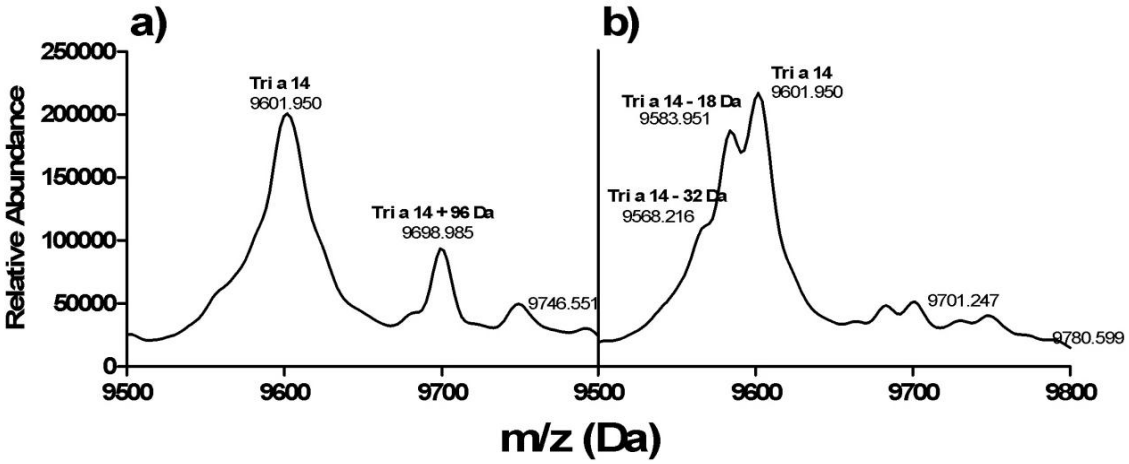


Fig. 6.4: MALDI – ToF – MS of Tri a 14 heated at 90°C (a) and 121°C (b).

6.3.2 Simulated gastroduodenal digestion of thermally treated LTPs

BLG was chosen to establish the effect of thermal processing on LTPs. BLG is denatured at 80°C and loses its helical structure leading in formation of β-sheets (Seo et al., 2010), therefore a fully denatured state was obtained at 121°C. A small proportion of denatured BLG got digested by pepsin while most of it retained after gastric proteolysis. There are several digestion products that can be seen on the gel below the BLG band ranging from 2 – 15 kDa (Fig. 6.5).

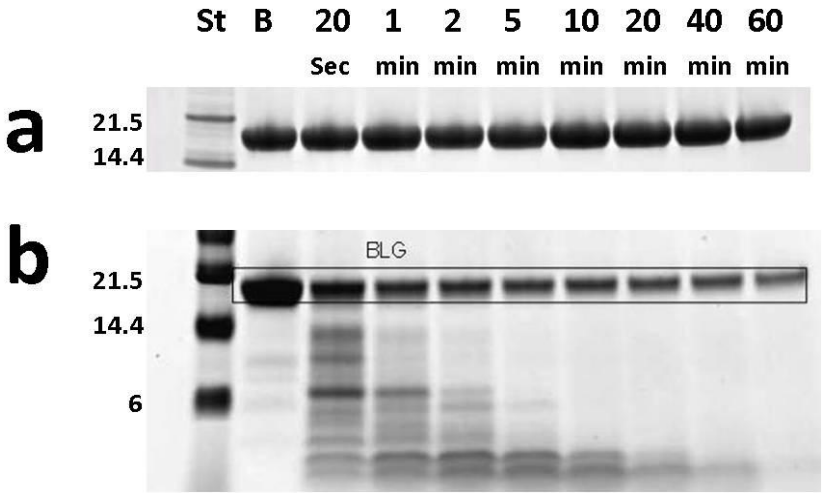


Fig.6.5: SDS-PAGE of the gastric digestion of unheated BLG (a) and heated at 121°C (b) performed under reduced conditions. "St" represent standard molecular weight markers lane while "B" represents blank lane in the absence of pepsin.

On subjection to duodenal proteolysis, it was found that the rate of digestibility of denatured BLG is faster than the native BLG. The heat treated denatured BLG completely digested after 15 minutes of digestion while it persisted until 30 minutes of digestion in its native state. The digestion products however are difficult to compare as they appear in the same range as appeared in the digestion of native BLG with very low abundance (Fig. 6.6).

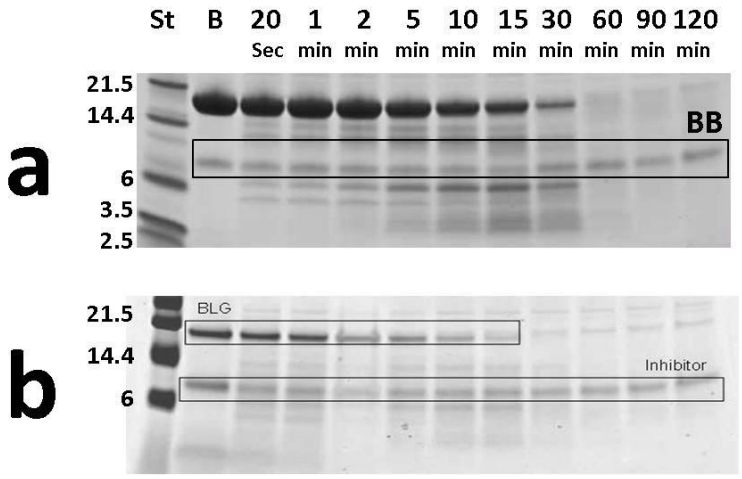


Fig 6.6: SDS-PAGE of duodenal digestion of unheated BLG (a) and heated BLG at 121°C (b). BB stands for Bowman birk inhibitor. "St" stands for standard molecular weight marker lane while "B" stands for blank lane without enzymes.

Pru p 3 was found to be digested slowly into two small peptides under gastric phase of digestion. Pru p 3 completely disappeared after 60 minutes of digestion under physiological conditions as observed by SDS-PAGE (Fig. 6.7) and MALDI – ToF – MS (Fig. 6.8 c).

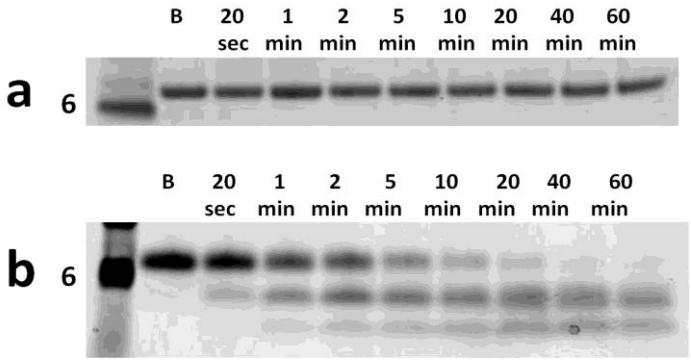


Fig. 6.7: SDS-PAGE of the gastric digestion of unheated Pru p 3 (a) and heated at 121°C (b) performed under reduced conditions. "B" represents blank lane in the absence of pepsin.

The samples obtained were analysed by MALDI – ToF – MS for the masses of the peptides obtained. After heating and dropping the pH to 2.5 in simulated gastric fluid prior to gastric digestion, Pru p 3 showed a sharp peak corresponding to intact Pru p 3 along with additional peaks corresponding to removal of 32 Da, 64 Da and 96 Da (Fig. 6.8 a). This might be due to loss of one, two and three sulphur atoms from the protein in the form of SO_2 as described above. During the gastric digestion phase, two major peptides were observed which can be seen below the Pru p 3 band on the SDS-PGE gel corresponding to molecular weight 5416.08 and 3690.93 Da, the sequence of which could not be identified on the basis of the intact mass of the peptide only (Fig. 6.8 b, c).

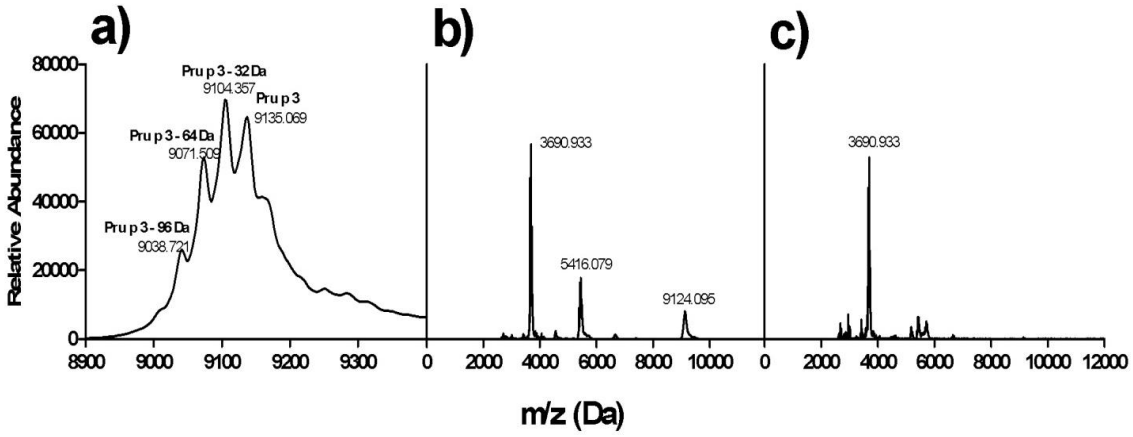


Fig. 6.8: MALDI – ToF – MS of heat treated Pru p 3 before gastric digestion (a), after 2 minutes (b) and 60 minutes of gastric digestion (c).

After 60 minutes of gastric digestion of Pru p 3, the MALDI – ToF – MS did not show any intact protein but only a single sharp peak corresponding to unidentified molecular weight 3690.93 Da. This is inconsistent with the SDS-PAGE results where after 60 minutes of digestion, at least two major bands can be seen corresponding to the digestion products of Pru p 3.

In duodenal phase of digestion, the digestion products of Pru p 3 by pepsinolysis completely disappeared after 30 minutes of digestion. The high molecular weight peptide disappeared even faster within 2 minutes of digestion while low molecular weight peptide persisted until 30 minutes of digestion (Fig. 6.9).

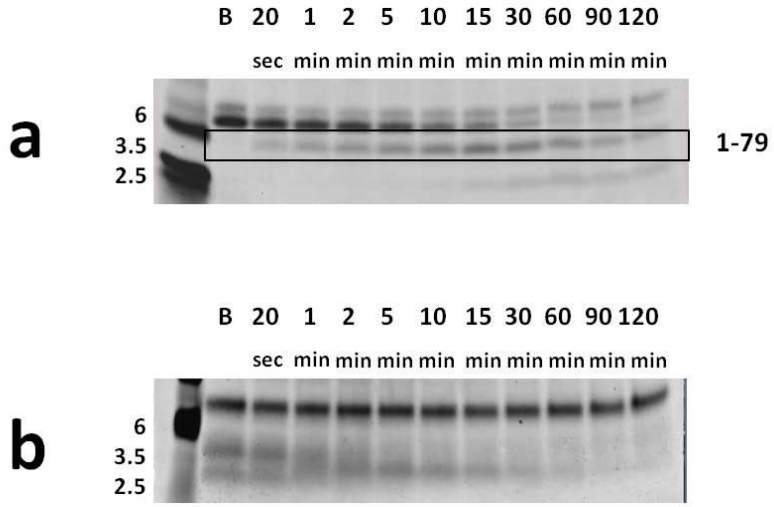


Fig.6.9: SDS-PAGE of duodenal digestion of unheated Pru p 3 (a) and heated Pru p 3 at 121°C (b). Inhibitor is also marked in (b) while it runs just above the Pru p 3 band in (a). "B" stands for blank lane without enzymes.

The MALDI – ToF – MS of Pru p 3 pepsinolysis products before duodenal digestion (Fig. 6.10 a) is identical to the MALDI – ToF – MS of 60 minutes digestion in gastric phase (Fig. 6.8 c). The spectrum consists of a single unidentified peak of molecular weight 3690.93 Da together with low

abundance unidentified peaks corresponding to molecular weight 5436 and 7844 Da. The MALDI – ToF – MS spectrum is inconsistent with the SDS-PAGE results (Fig.6.9 b; lane B) where the same sample shows two distinct bands corresponding to digestion products of pepsinolysis of heat treated Pru p 3 excluding the inhibitor band.

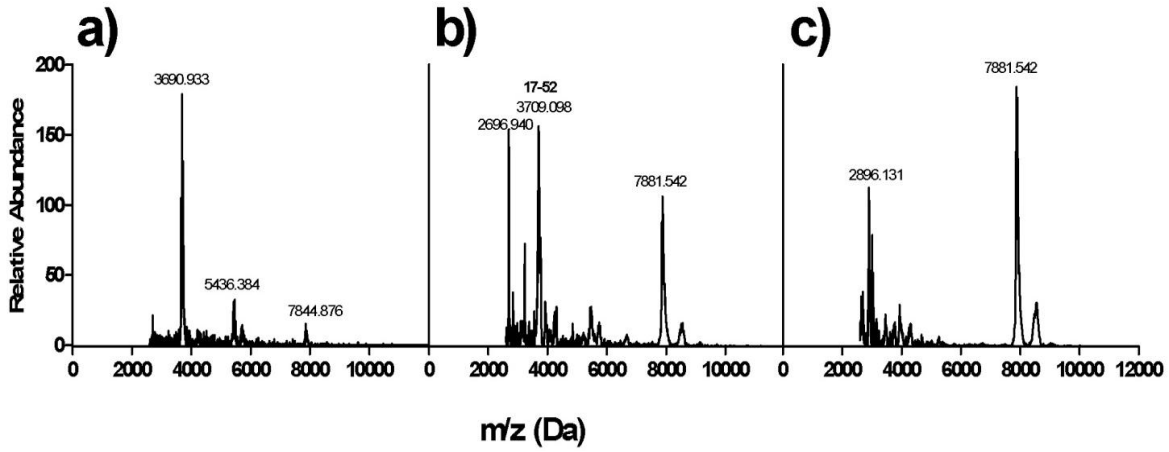


Fig. 6.10: MALDI – ToF – MS of heat treated Pru p 3 before duodenal digestion (a), after 2 minutes (b) and 120 minutes of duodenal digestion (c).

The MALDI ToF MS of two minutes of duodenal digestion of heat treated Pru p 3 (Fig. 6.10 b) shows few sharp peaks corresponding to molecular mass 7881.54 Da corresponding to Bowman Birk inhibitor and two more peaks of molecular weight 3709 Da corresponding to residues 17-62 and one unidentified peak of molecular mass 2696 Da. The peptide 17-52 represents the residues found that of Q9LED1. The result is consistent with that of SDS-PAGE (Fig. 6.9 b; 2 min. lane) where the lane contains two major bands corresponding to inhibitor and the pepsinolysis product of Pru p 3.

The spectrum of MALDI – ToF – MS of 120 minutes of duodenal digestion of heat treated Pru p 3 shows one unidentified major peak of 7881.54 Da corresponding to Bowman Birk inhibitor together with one unidentified peak of 2896 Da. The result is consistent with SDS-PAGE where none of the digestion product left after 120 minutes of digestion in duodenal phase.

Tri a 14 was found to be considerably stable during the gastric digestion phase as compared to Pru p 3 and was digested into large stable peptide very slowly. The fragment increased in abundance after 20 minutes of digestion (Fig. 6.11 b).

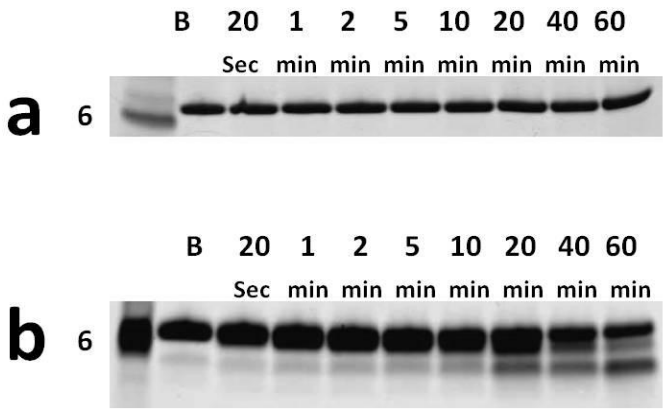


Fig. 6.11: SDS-PAGE of the gastric digestion of unheated Tri a 14 (a) and heated at 121°C (b) performed under reduced conditions. "B" represents blank lane in the absence of pepsin.

Tri a 14 was found to be in various degrees of sodiation before subjecting to gastric proteolysis as observed in MALDI – ToF – MS showing additional peaks corresponding to 23, 46 and 69 Da increase in molecular mass as a result of dropping the pH from 7 to 2.5 in SGF which is a prerequisite of gastric proteolysis (Fig. 6.12 a).

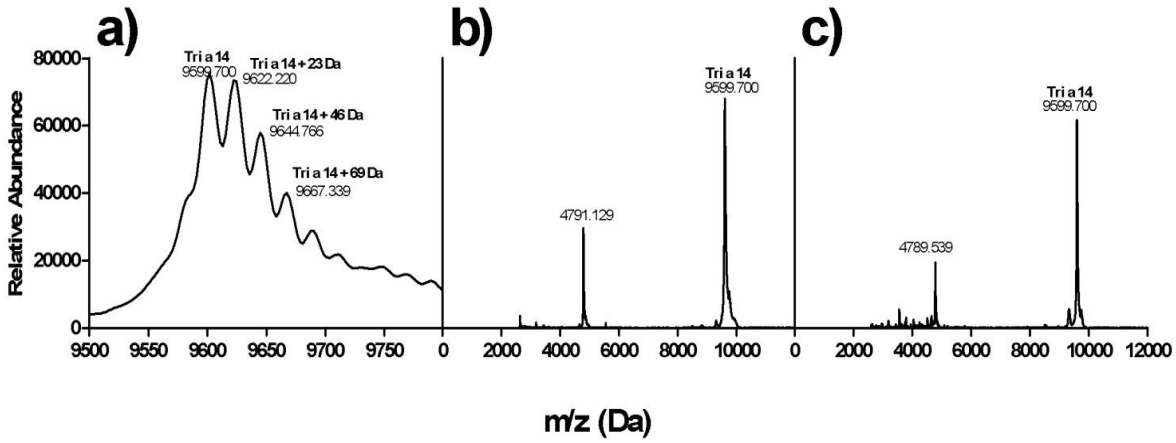


Fig. 6.12: MALDI – ToF – MS of heat treated Tri a 14 before gastric digestion (a), after 2 minutes (b) and 60 minutes of gastric digestion (c).

The MALDI – ToF - MS spectrum of two minutes of gastric digestion of Tri a 14 showed a single sharp peak corresponding to intact Tri a 14 (Fig. 6.12 b) while additional peaks corresponding to addition of 23 and 46 Da similar to the protein prior subjecting to gastric proteolysis, were also seen (data not shown). The spectrum also contained a peak of Mr 4791 Da which was unidentified but may reflect to small digestion product seen on SDS-PAGE (Fig. 6.11). At the end of gastric phase of digestion, Tri a 14 still showed a single sharp peak corresponding to native intact Tri a 14 with an additional unidentified peak of Mr 4789 Da. The result is consistent with the SDS-PAGE result

(Fig. 6.11 b; 60 min. lane) where a high abundant digestion product can be seen below the Tri a 14 band.

Tri a 14 was found to be extremely slowly digested until 240 minutes of duodenal digestion with the large proportion of the protein remaining intact by the end of the digestion phase. There might be some digestion fragments with very low abundance which could not be seen on SDS-PAGE (Fig. 6.13).

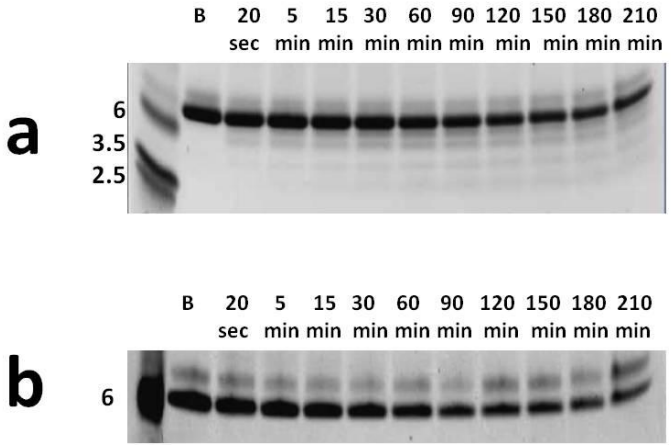


Fig. 6.13: SDS-PAGE of duodenal digestion of unheated Tri a 14 (a) and heated Tri a 14 at 121°C (b). Inhibitor runs just above the Tri a 14 band. "B" stands for blank lane without enzymes.

The MALDI – ToF – MS spectrum of Tri a 14 before duodenal digestion (Fig. 6.14 a) contains a single sharp peak of 1623.11 Da corresponding to an unidentified peptide. The spectrum is devoid of any intact Tri a 14 and hence is inconsistent with the SDS-PAGE result (Fig. 6.13 b; lane B) where Tri a 14 is seen in a high abundance band.

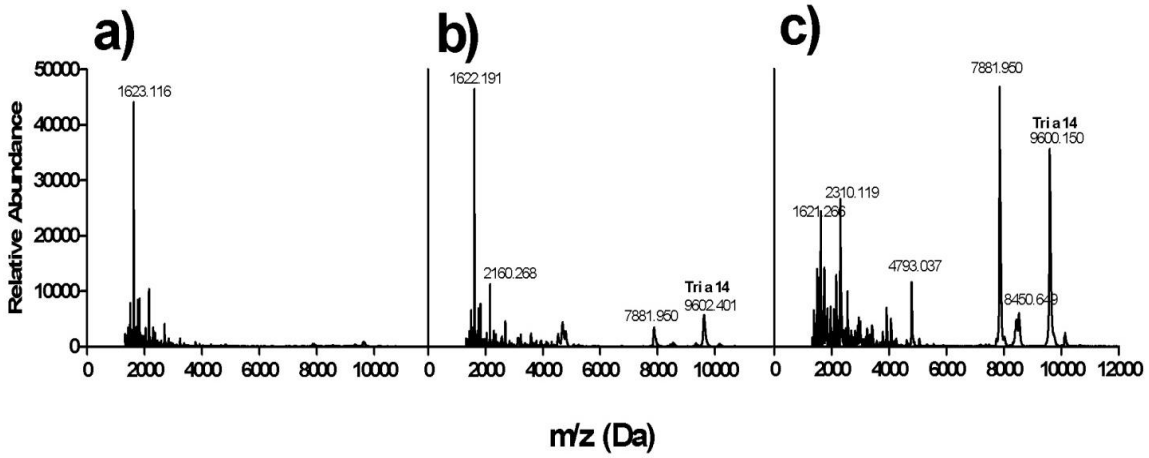


Fig. 6.14: : MALDI – ToF – MS of heat treated Tri a 14 before duodenal digestion (a), after 2 minutes (b) and 120 minutes of duodenal digestion (c).

The MALDI – ToF – MS spectrum of Tri a 14 after two minutes of duodenal digestion (Fig. 6.14 b) contains a very small peak corresponding to intact Tri a 14 together with some small unidentified peaks and a very high abundant unidentified peak of 1622.19 Da.

The MALDI – ToF – MS spectrum of Tri a 14 after 120 minutes of duodenal digestion contains several unidentified peaks of 4793.04, 2310.12 and 1621.26 Da. The highest abundant peak corresponding to molecular mass 7881.19 Da correspond to Bowman Birk inhibitor along with a high abundant Tri a 14 peak. The result is consistent with SDS-PAGE result (Fig. 6.13 b; 120 min lane) where two most abundant bands are due to Bowman Birk inhibitor and intact Tri a 14. A sequence comparison of Pru p 3, Tri a 14, Mal d 3 (apple LTP) and Vit v 1 (grape LTP) is given in Fig. 6.15.

```
Pru p 3:   ITCGQVSSSLAPCIPYVRGGGAVPPACCNGIRNVNNLARTTPDRQAACNCLKQLS 55
Mal d 3:   ITCGQVTSSLAPCIGYVRSGGAVPPACCNGIRTINGLARTTADRQTACNCLKNLA 55
Vit v 1:   LSCGDVATQLAPCINYLRSAGPLPVACCNGVKNLKNSAATTQDRRTACKCLINAS 55
Tri a 14:  IDCGHVDSLVRPCLSYVQGGPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIA 55

Pru p 3:   ASVPGVNPNNAAALPGKCGVSIPYKISASTNCATVK 91
Mal d 3:   GSISGVNPNNAAGLPGKCGVNVPYKISTSTNCATVK 91
Vit v 1:   KSISGVNFGLAAGLPGKCGVNIPYKISPSTNCDQVN 91
Tri a 14:  RGIHNLNEDNARSIPPKCGVNLPYTISLNIDCSRV- 90
```

Fig. 6.15: Sequence alignment of various LTPs taken account in this study.

6.3.3 Simulated gastro-duodenal digestion of native food matrices

6.3.3.1 Digestion of Pru p 3 in peach peel

Pru p 3 was found to be stable in the gastric phase of digestion in both soluble supernatant and insoluble pellet within peach peel as seen on SDS-PAGE (Fig. 6.16). No digestion product was seen on SDS-PAGE and the protein survived after 120 minutes of digestion in both soluble supernatant and insoluble pellet. In duodenal phase of digestion, Pru p 3 was found to be digested very slowly which is difficult to see on SDS-PAGE in either soluble supernatant or insoluble pellet but was observed in the immunoblots of the selected samples. Therefore, further analysis of the gel could not be carried out due to the poor resolution of the bands on the gel. Other proteins of interest include peach thaumatin like protein with a molecular weight of 28 kDa appearing only in the soluble supernatant of the gastric phase of digestion (Fig. 6.16 a) which was found to be stable throughout the gastric digestion phase. The result was confirmed by immunoblotting of the digests (Fig. 6.17).

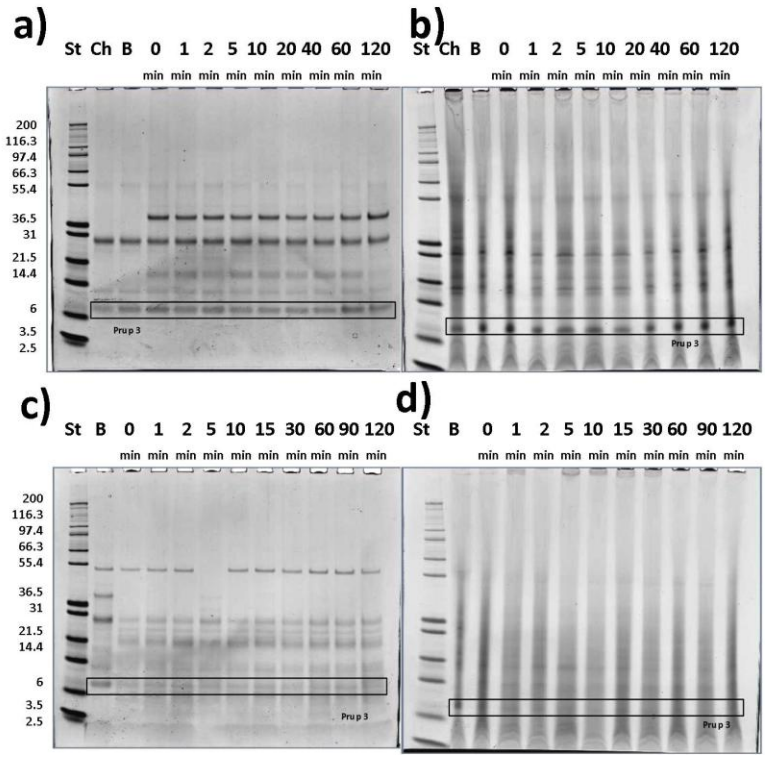


Fig. 6.16: SDS-PAGE of the supernatant (a) and pellet (b) of gastric digestion and supernatant (c) and pellet (d) of duodenal digestion of peach under simulated conditions. St represents the standard molecular weight marker lane, Ch represents material after oral digestion, B represents material before subjection to proteases while location of Pru p 3 within the gel is represented by a rectangular box on the gel.

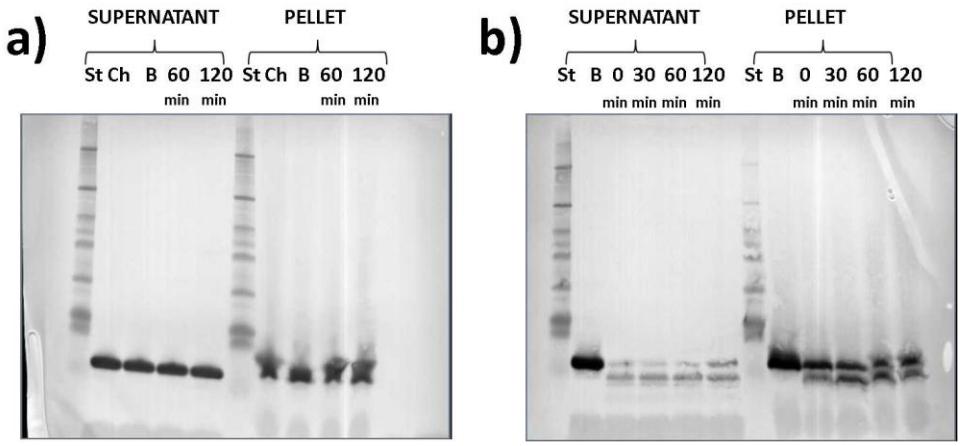


Fig. 6.17: Immunoblots of simulated gastric (a) and duodenal (b) digestion of peach peel. The selected time points are mentioned in the figure legend at the top. Soluble supernatant and insoluble pellet is also mentioned. St represents standard molecular weight marker lane, Ch represents material after oral digestion while B represents material before proteolysis.

Immunoblots of the peach peel digests also confirmed that Pru p 3 remained intact during gastric digestion until a time course of 120 minutes in both soluble supernatant and insoluble pellet. No digestion product was seen neither in the supernatant nor in pellet as evidenced by a single band. In case of digestion during duodenal phase, Pru p 3 was digested very slowly into one major product which appeared below the Pru p 3 band. After 120 minutes of digestion, nearly half of the Pru p 3 remained intact while other half digested into one large peptide as seen in immunoblot (Fig. 6.17 b). This proportion is based on visual observation only since the image could not be analyzed by Totallab.

6.3.3.2 Digestion of Tri a 14 in wheat flour and its fractions

The digestibility of Tri a 14 was investigated in various commercial flour, bran and white flour along with the cultivar variety "Cadenza" included in this study. Tri a 14 was found to be stable to gastric proteolysis in all forms of the matrices included in this study (Fig. 6.18). No digestion product was seen on SDS-PAGE.

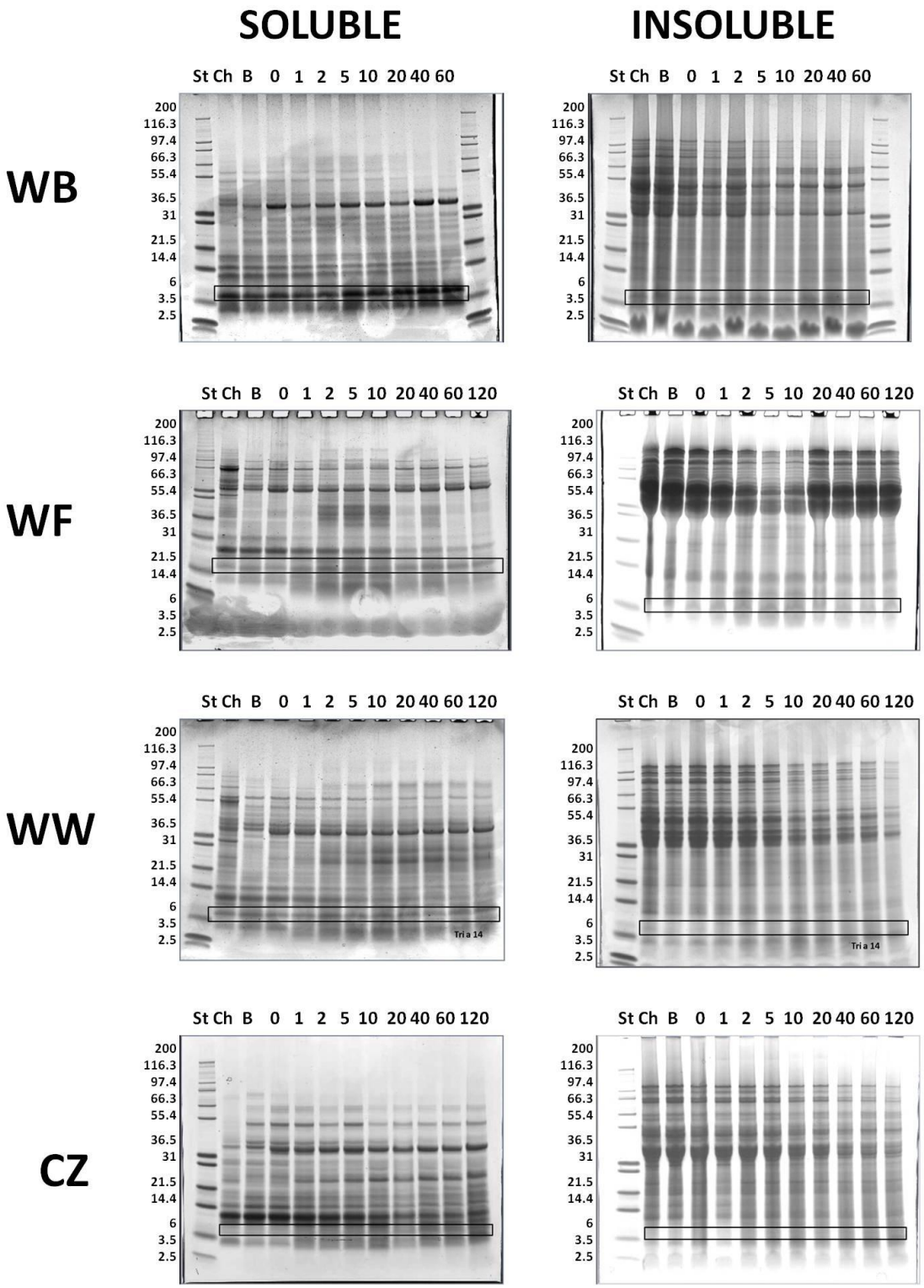


Fig. 6.18: SDS-PAGE of the simulated gastric digestion of Tri a 14 in various flours and its fractions. **WB, WF, WW** and **CZ** represent wheat bran, white flour, whole wheat flour and cadenza flour respectively.The soluble supernatant is given in **SOLUBLE** column while insoluble pellet is given in **INSOLUBLE** column. Position of Tri a 14 is shown in boxes on the gels.

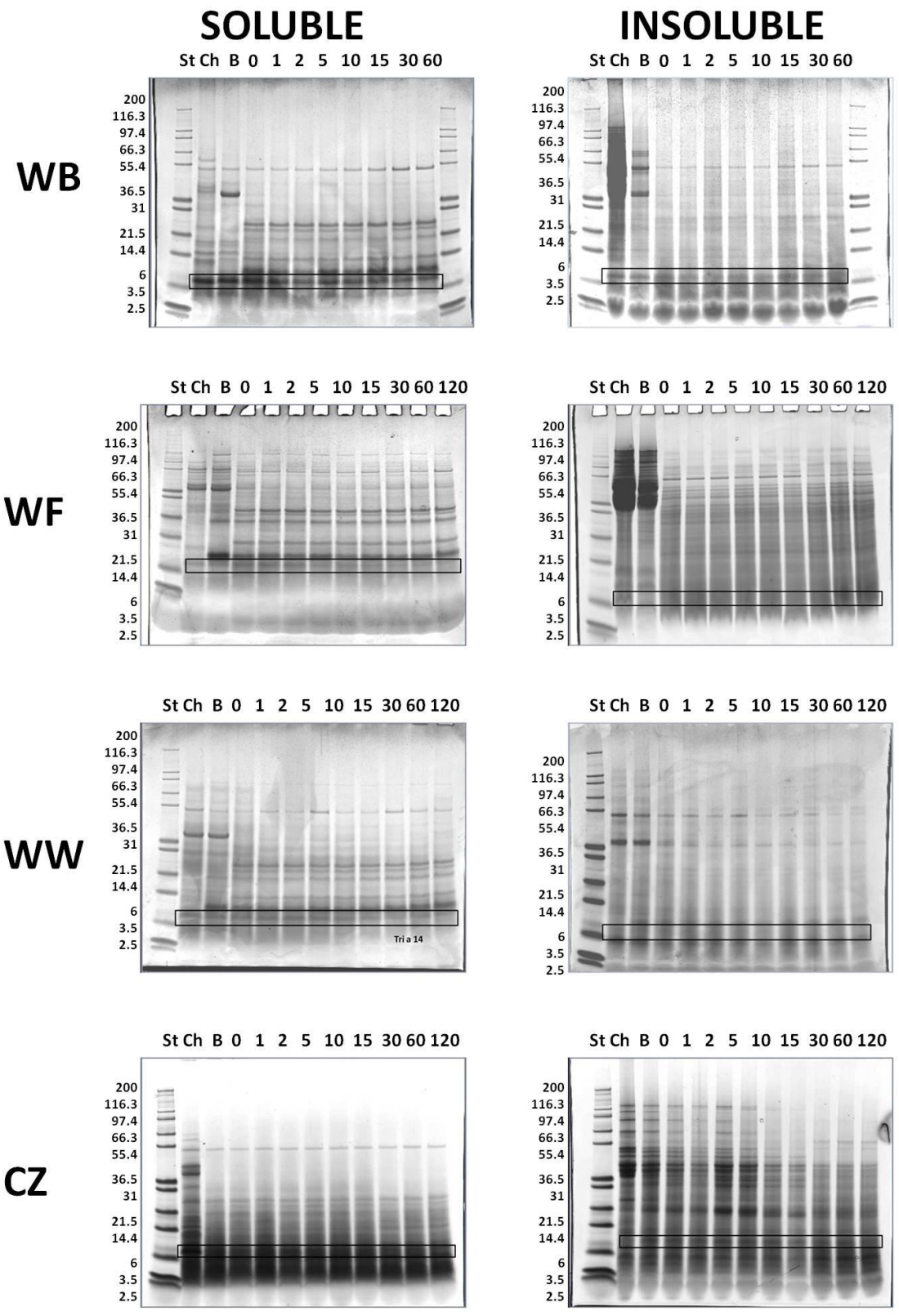


Fig. 6.19: SDS-PAGE of the simulated duodenal digestion of Tri a 14 in various flours and its fractions. **WB, WF, WW** and **CZ** represent wheat bran, white flour, whole wheat flour and cadenza flour respectively.The soluble supernatant is given in **SOLUBLE** column while insoluble pellet is given in **INSOLUBLE** column. Position of Tri a 14 is shown in boxes on the gels.

Tri a 14 was found to be stable in both gastric and duodenal phase of digestion in both soluble supernatant and insoluble pellet (Fig. 6.18 and 6.19). No digestion fragment was identified on SDS-PAGE mainly because of other interfering proteins found in the material. In the Case of cadenza flour, the material initially was either devoid of Tri a 14 or was present at extremely low level since Tri a 14 band could not be identified on SDS-PAGE gel. Tri a 14 was found in high levels in either wheat bran or whole wheat flour and showed stability to digestion to time duration of 120 minutes in both gastric and duodenal phase of digestion. Tri a 14 was also found to be very soluble in the soluble supernatant and was found to be released from the material at the start of digestion. Further characterization and confirmation of this stability needs extensive mass spectrometric analysis.

6.3.4 Simulated gastro-duodenal digestion of heat processed food matrices

6.3.4.1 Digestion of heat processed peaches

Pru p 3 was found to be stable to gastric proteolysis after heat treatment at 121°C for 15 minutes within peel and pulp. This is shown by SDS-PAGE of both soluble supernatant and insoluble pellet where Pru p 3 band is seen after 120 minutes of digestion (Fig. 6.20).

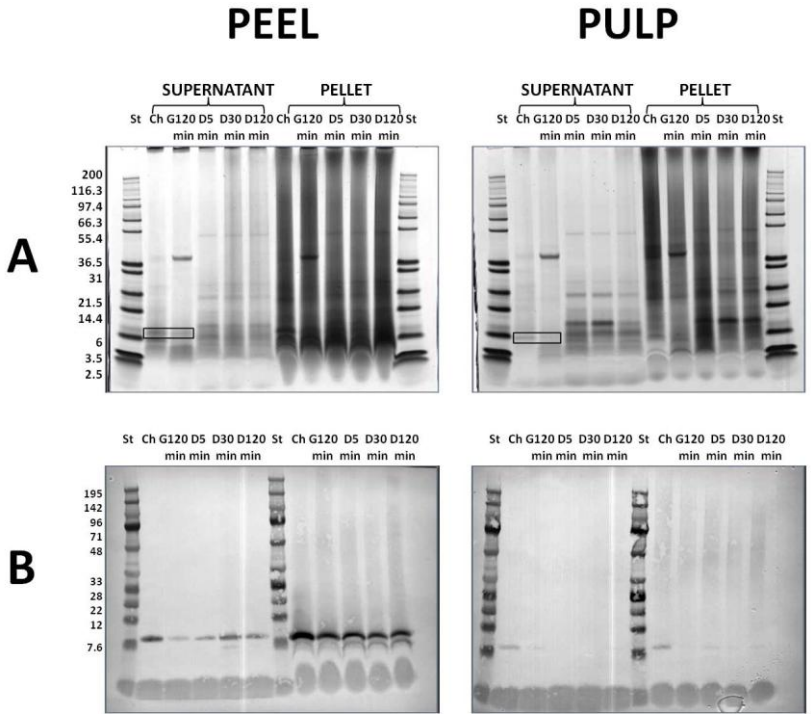


Fig. 6.20: SDS-PAGE of the simulated gastroduodenal digestion of peach peel and pulp (A) and immunoblots of the same samples (B). Location of Pru p 3 is also shown in boxes. G and D represents gastric and duodenal phase respectively.

Pru p 3 was slowly digested into a large stable peptide in the duodenal phase of digestion in both peel and pulp as seen on SDS-PAGE (Fig. 6.19 AI and AII). Immunoblots also confirmed this result. Pru p 3 was found to be in very low abundance in soluble supernatant of both peel and pulp

while pellet contained considerably high level. After 120 minutes of duodenal digestion, Pru p 3 was found to be mostly intact with a very small proportion of it digested into large stable peptide in peel while pulp did not show this behaviour mainly because of very low level of Pru p 3. The effect of heating in syrup did not show much difference with the similar stability of Pru p 3 in peel and very low abundance in pulp (data not shown).

6.3.4.2 Digestion of bread

Tri a 14 was found to be stable in both gastric and duodenal phase of digestion n the soluble supernatant of crumb of the bread after 120 minutes of digestion in each phase (Fig. 6.21). The result is based on the presence of similar abundance of Tri a 14 band at the end of digestion phase.

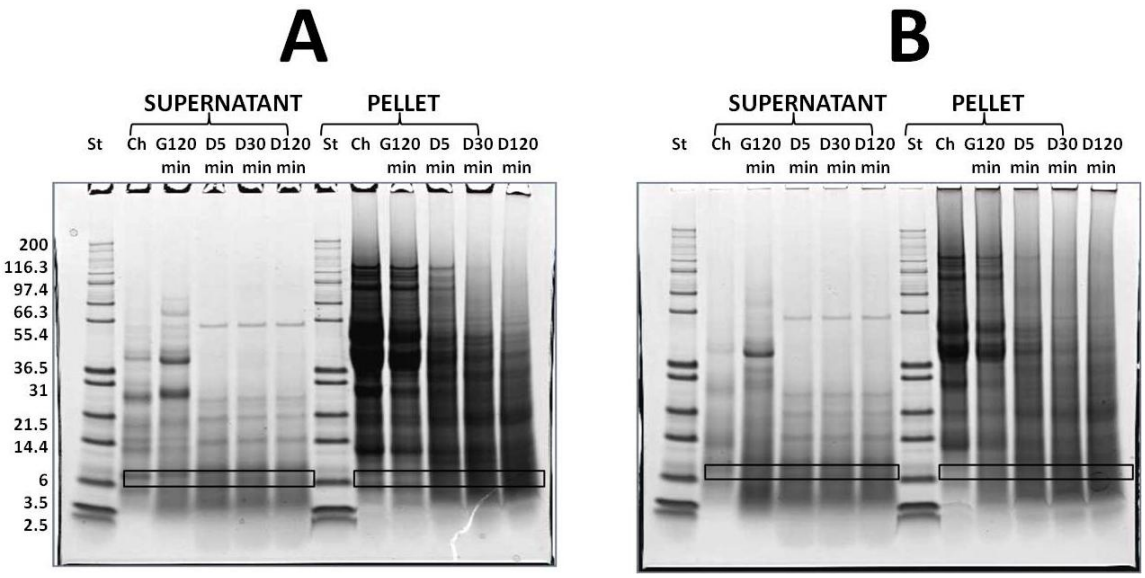


Fig.6.21: SDS-PAGE of the simulated gastric (represented by G) and duodenal (represented by D) digestion of crumb (A) and crust (B) of bread. St represents standard molecular weight marker lane while Ch represents material after oral digestion. Location of tri a 14 is also shown in the boxes.

Tri a 14 could not be identified in the SDS-PAGE of the both soluble supernatant and insoluble pellet of crust digestion and insoluble pellet of crumb digestion. This is because of the oxidized components of wheat and the Maillard reaction products of the Lys in wheat proteins and amylolytic products of starch generated as a result of digestion or very low abundance in flour need 2D gels.

6.4 Chapter conclusion

It is already established that Pru p 3 is the major food allergen in Southern Europe which cross reacts with other *Rosaceae* fruits (Asero et al., 2002, Sánchez-Monge et al., 1999, Diaz-Perales et

al., 2000). Despite the fact that Pru p 3 is very similar to Mal d 3 in both primary and secondary structure, Pru p 3 showed irreversible denaturation in contrast with Mal d 3 (Sancho et al., 2005). On heating and cooling Pru p 3, it was clear from the data that Pru p 3 lost its secondary structure and was unfolded. On the other hand, Mal d 3 also showed a loss in its secondary structure on heating, which was refolded to a structure similar to its native fold on cooling. Therefore, this comparison states that Pru p 3 is less stable than Mal d 3. This result is also in contrast with the fact that Pru p 3 contains 9 residues of proline as compared to Mal d 3 containing only 6 residues of this amino acid (Fig. 6.15). According to proline rule, proteins with higher number of proline residues are expected to be more thermostable (Watanabe et al., 1997). Therefore, Pru p 3 should be more thermostable than Mal d 3 having higher proline residues, whereas our results suggest that Pru p 3 is less stable than Mal d 3. This is already established that resistance to processing conditions such as thermal process may be the reason for this class of allergens to be allergenic (Breiteneder and Mills, 2005b). However, Pru p 3 is reported to be more allergenic being the primary sensitizer than Mal d 3, which cross reacts with Pru p 3. These results thus suggest further studies to make clear conclusions whether the structural stability of LTPs are directly associated with their potential of allergenicity. Tri a 14 is very distinct in primary structure to Pru p 3 containing 16 charged residues as compared to Pru p 3 with only 8 charged residues. Therefore, the protein has higher intra- and intermolecular interactions making the structure rigid to denaturation. Therefore, a high stability to thermal denaturation was observed here.

The heat treatment to Pru p 3 mainly appeared in sodiation of protein along with removal of SO_2 as a result of oxidation (Sancho et al., 2005). This loss of "S" from cysteine destroyed the tertiary structure of protein which may alter the flexibility of the helices resulting higher mobility of residues. This higher mobility may also support the protein – enzyme interaction which might be the reason for the protein being less stable in gastric compartment. The structural change allowed protein to be more digestible by pepsin in contrast with the fact that LTPs are pepsin resistant (Asero et al., 2000). This is due to the fact that major structural stability of LTPs are considered due to the presence of conserved cysteins which form four intramolecular disulphide bridges making the protein a compact globular structure. After losing at least one sulphur, this compact structure is lost resulting the protein more susceptible to pepsinolysis. Since Tri a 14 showed little denaturation to heat treatment, while a proposed dehydration and loss of a single sulphur in small population of protein occurred, the digestibility of this protein by pepsin remained very slow leaving 50% of the protein intact until 60 minutes of digestion. This 50% population may be those molecules which remained stable to heat and conserved with four disulphide bridges. In duodenal condition, Tri a 14 showed more resistance than its native form. this might be due to the fact that

the heat treatment at 121°C decreased the helicity of LTP, a major factor involved in susceptibility to digestion into increased β-sheets, the hypothetical more resistant to digestion than α-helices. This result explains that partial thermal denaturation of this protein stabilizes it and makes it more resistant to duodenal digestion.

The effect of food matrix is studied in this chapter. The digestibility of Pru p 3 in its native peel showed more resistance to digestion than its purified form. This result may be due to the fact that oral chew does not homogenize the peel components and hence, most of the protein remains intact within the undisrupted tissues and hence, have slower access to enzymes. Since in the gastric phase of digestion, Pru p 3 remains intact, this remains intact when it is within the peel. However, during duodenal phase of digestion, Pru p 3 showed higher digestibility in its purified form than within peel. Besides the physical intereferences, the peel matrix components are equally available to enzymes also to modify them and affect their activities which might be the case in this higher resistance of Pru p 3 in peel. Tri a 14 being very stable in the purified form to digestion remained stable in its native matrix among various forms of flour.

The effect of heat treatment also confirmed that food matrix plays a very important role in the digestibility of LTPs. This is demonstrated by the fact that heat treated purified Pru p 3 was digested by pepsin within 60 minutes of digestion while it survived even 120 minutes of pepsinolysis after heat treatment within the peel. This might be due to the fact that peach peel contains a lot of matrix components such as carbohydrates, pectin, polyphenols which all can react Pru p 3 and stabilize against heat denaturation. Similarly, these matrix components are also equally available to pepsin to modify it and affect its activity. Therefore, Pru p 3 survived even 120 minutes of gastric digestion within heat treated peel which in case of purified Pru p 3, disappeared after 60 minutes of digestion. The present study thus confirms that food matrix is very important to understand the behaviour of allergens within the food system and a careful study is required to understand the possible mechanism underlying these adverse reactions. This matrix is also important in the way, how it delivers the processed LTPs to immune system, modified, digested or intact.

7 Effect of ligand binding and food matrix on IgE binding of lipid transfer proteins

7.1 Introduction

Peach allergy is the most prevalent food allergy in Spain and relatively common allergic fruit in Europe and USA. The allergy to peach is mediated by IgE and hence, classed as true food allergy (Cuesta-Herranz et al., 1998a). The food biological activity is a quantitative measure of allergenic potential of a food. This is standardized with allergen solution followed by measuring allergenic potential of food extracts containing the same allergen. Peach biological activity has been studied as a model (Cuesta-Herranz et al., 1998b) and the method developed may be a helpful tool to standardize other allergic foods and their allergenicity.

Pru p 3 has been hypothesized to be responsible for respiratory allergy being present in peach tree leaves and positive skin prick and bronchial provocation test (García et al., 2004). Similarly, Pru p 3 has been identified as the major allergen in peach allergic population in Spain (Fernández-Rivas et al., 2003). LTPs from *Rosaceae* have been shown to crossreact with each other, while Pru p 3 was found to be the primary sensitizer. This phenomena is due to the presence of common epitopes in the members of LTP family to *Rosaceae* fruits (Borges et al., 2008). Plane pollen allergens have been shown to be immunologically related to Pru p 3. Plane pollen allergic patients co-allergic to Pru p 3 identified Pla a 3 as the major LTP allergen while plane pollen allergic patients without Pru p 3 allergy showed Pla a 3 as minor allergen (Lauer et al., 2007). In an opposite way, peach allergic patients showed allergy to pollens from grass such as *Lolium* and *Phleum* followed by trees such as *Olea* and weeds pollen such as *Plantago* as determined in Spanish population (Cuesta-Herranz et al., 1999). The grass pollen sensitization followed by allergy to peach is clinically related to peach profilin, an actin binding protein and is cross reactive to Bet v 2 (Rodríguez-Perez et al., 2003). Oral allergy syndrome (OAS) to peach was found to be cross reactive to other Prunoideae members such as cherry, apricot and plum because of the presence of a 13 kDa allergenic protein found all of them while 14 kDa protein in peach and 30 kDa protein in cherry was also found to be cross reactive to grass and birch pollen (Pastorello et al., 1994).

LTPs have been proposed as model “true” food allergens due to their resistance to proteolysis, ability to sensitize through oral route and association with severe clinical symptoms. Pru p 3 is stable at neutral and acidic pH (3.0) while becoming denatured only at high temperature (95°C) (Gaier et al., 2008). These characteristics ensure the survival of immunologically intact form able to bind IgE after oral exposure (Salcedo et al., 2008). Thus simulated gastroduodenal digestion and

subsequent IgE recognition of the digestion product showed that intact Pru p 3 and its high molecular weight digestion products only are recognised by IgE (Cavatorta et al., 2010).

Both the natural and recombinant forms showed equivalent structural and immunological properties (Díaz-Perales et al., 2003). In the case of Rosacea fruits, LTPs have been found to be abundant in peel (Borges et al., 2006). The Pru p 3 accumulation is dependant upon the variety of the plant. For example, cv Royal Gem has been shown to accumulate more Pru p 3 than cv Rita Star. Commercial harvesting followed by storage at room temperature improved yield (Botton et al., 2006).

Pru p 3 comprises four helices with three peptides identified as major epitopes corresponding to residues 23-36, 39-44 and 80-91 while Arg39, Thr40, Arg44, Lys80 and Lys91 have been identified as the potential residues responsible for IgE recognition (García-Casado et al., 2003). Cross-reactivity of IgE between Pru p 3 and Tri a 14 in the case of Baker's asthma patients was based on presence of common epitopes corresponding to residues 31-40 and 71-80 (Tordesillas et al., 2009). The IgE recognition of reduced and alkylated Pru p 3 was diminished (Toda et al., 2011).

In the last section of the study, the samples generated during the study were assessed for their IgE binding capacity to gain an indication of the potential biological relevance in food allergy. This work was undertaken as part of a visit to the laboratory of Prof Ronald van Ree at the Academic Medical Centre (AMC) Amsterdam.

7.2 Methods

7.2.1 Ligand binding and simulated gastro-duodenal digestion of Pru p 3

Pru p 3 was purified as described in chapter 3. The ligand loading with linoleic acid was performed as described in section 5.2.2 and the simulated gastro-duodenal digestion was performed as described in section 5.2.3.

7.2.2 Thermal processing and gastro-duodenal digestion of Pru p 3

Purified Pru p 3 was subjected to heat treatment as described in section 6.2.1.1. The heat treated Pru p 3 was then subjected to simulated gastro-duodenal digestion as described in section 2.2.3.

7.2.3 Digestion of peach peel

Fresh peaches were obtained from a local market in Norwich. Peel was removed by using a domestic knife keeping the thickness as 2-3 mm. Model chew and simulated gastroduodenal digestion of the peel was followed as described in section 2.2.4.

7.2.4 SDS-PAGE and immunoblotting

In case of heat processed Pru p 3, the gastric digest after 60 minutes and duodenal digest after 120 minutes were run on 2D SDS-PAGE gel (NuPAGE, Invitrogen, Paisley, UK). In the case of Pru p 3 loaded with linoleic acid, only the duodenal digest after 120 minutes was electroblotted. For each gel, 175 µg protein was loaded into a single large slot and SDS-PAGE carried out as described in section 2.1.2. At the end of a run, the gel was electroblotted onto nitrocellulose membrane and blocked with skimmed milk powder as described in section 2.4.4. The blocked membrane was air dried overnight at room temperature and stored at -20°C until required.

In the case of peach peel digests, the oral digest after 2 minutes, gastric digest after 60 minutes and duodenal digest after 120 minutes were separated into supernatant and pellet and SDS-PAGE carried out with 2D gel (NuPAGE, Invitrogen, Paisley, UK) as described in section 6.2.4. After SDS-PAGE, the gel was electroblotted on nitrocellulose membrane and blocked with skimmed milk powder as described in section 2.4.4. The blocked membrane was air dried overnight at room temperature and stored at -20°C until required.

7.2.5 Patients' sera

Six different sera out of which five allergic to peach were kindly provided by Dr. Ronald van Ree (AMC, University of Amsterdam, Amsterdam, Netherlands) namely PF194, PF227, P0705, P0725 and P0715. The non-atopic control serum namely PF201 was used as negative control. The IgE titre information provided with sera is given in Table 7.1.

7.2.6 IgE binding

For IgE binding, the nitrocellulose membrane was cut into 3 mm strips vertically parallel to the direction of running so that Pru p 3 content in a single strip was 5 – 8 µg (excluding for peach peel samples with unknown Pru p 3 content). Patient's serum (150 µL) was diluted into 3 mL of 0.5% (w: v) skimmed milk powder dissolved in PBST prior to use (see section 2.4.2.2). One strip of electroblotted membrane strip was placed in a 15 mL tube (BD Falcon tube, BD Biosciences, Oxford, UK) and 3.15 mL of the serum preparation added. The tube was incubated at 4°C overnight with gentle shaking. The next day, the strip was briefly rinsed seven times with PBST and tapped gently to remove excess buffer. Human anti-IgE raised in mouse conjugated with Odyssey IR Dye 800 CW (Li-Cor Biosciences, Lincoln, Nebraska, US) was diluted 1:2000 (v:v) in 0.5% (w: v) skimmed milk powder dissolved in PBST and 3 mL of this solution applied to the strip. The strip was incubated in dark at room temperature for 3 h with gentle shaking followed by rinsing briefly atleast seven times with PBST. The strip was tapped gently to remove excess buffer and dried at

room temperature in dark. The image of the strip was taken with Odyssey Infrared imaging system (Li-Cor, Lincoln, Nebraska, US).

7.3 Results

7.3.1 Patients' sera characteristics and identification to Pru p 3

Sera from two patients namely PF194 and PF227 were from plasmaforesis samples; hence original information regarding patient's characteristics was not available. The remaining three sera were maintained as a serum bank at the Academic Medical Centre, University of Amsterdam, Amsterdam with the IgE titres to peach (Table 7.1) determined by Phadia ImmunoCAP system (Uppsala, Sweden). Serum P0715 was the highest titre serum (160 kU/L) while serum P0705 with the lowest known titre (41.1 kU/L).

Table 7.1: characteristics of sera of patients allergic to peach

Serum number	Patient code	IgE titre (kU/L)	Other information
1	PF201	-	Non-atopic control
2	PF194	-	Plasmaforesis sample of Dutch patient (DA1) allergic to peaches
3	PF227	-	Plasmaforesis sample of Dutch patient (DA2) allergic to peaches
4	P0705	41.1	FAST serum bank*
5	P0725	28.4	FAST serum bank*
6	P0715	160	FAST serum bank*

* FAST is an EU-funded collaborative project co-ordinated by Prof. Ronald van Ree at AMC to develop immunotherapy strategies for peach allergy.

In the IgE immunoblot, Pru p 3 was well recognized by IgE from all sera from peach allergic patients, as shown in Fig. 7.1. Serum 4 showed the weakest IgE recognition of Pru p 3 while serum 6 having the highest binding. This result is not consistent with the ImmunoCAP anti-peach IgE titres which showed serum 5 had the lowest IgE titre while serum 4 having a slightly higher titre than serum 5. However, serum 6 recognition to Pru p 3 was consistent with its anti-IgE titre. Sera 2 and 3 also recognized Pru p 3, serum 2 showing higher recognition indicating probably this serum was likely to have a higher IgE titre than serum 2.

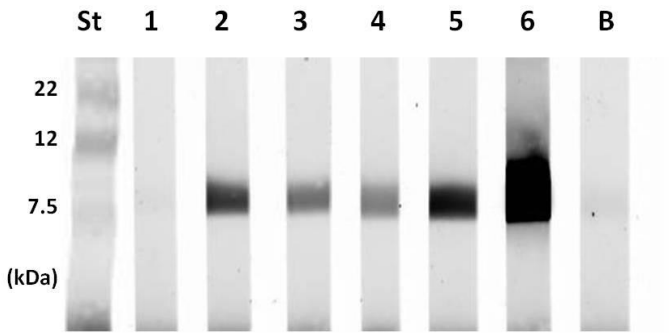


Fig. 7.1: IgE immunoblot of purified Pru p 3. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) as described in Table 7.1. Serum 1 was from a non-atopic control. B represents blot incubated with skimmed milk powder only.

7.3.2 Effect of ligand binding and simulated gastro-duodenal digestion of Pru p 3 on IgE reactivity

Pru p 3 remained intact and in well folded form after 60 minutes of gastric digestion (section 5.3.2) and others have shown it to retain its IgE-bindig capacity following simulated gastric digestion (Asero et al., 2000). Therefore, gastric digestion was excluded from this study. The duodenal digestion of Pru p 3 pre-dominantly appeared as peptides corresponding to residues 1-79 which appeared just below the Pru p 3 band on SDS-PAGE (Fig. 5.7 a) which further digested into two peptides, 1-39 and 40-79. The IgE from patients identified only Pru p 3 and its digestion product corresponding to residues 1-79 as shown by IgE immunoblot (Fig. 7.2).

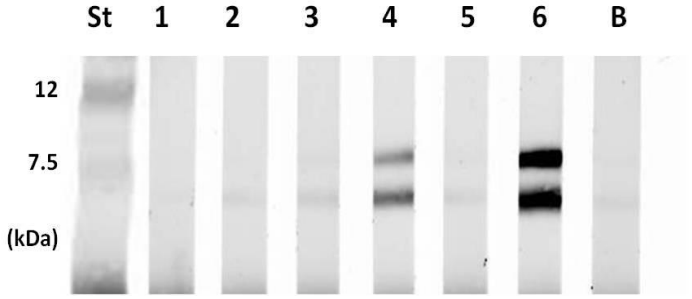


Fig. 7.2: IgE immunoblot of 120 minutes duodenal digestion of Pru p 3. St represents standard molecular weight markers. Lanes (1-6) represent patient sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

Serum 6 strongly identified Pru p 3 and its digestion product corresponding to residues 1-79 only. Surprisingly, serum 4 recognized both Pru p 3 and peptide 1-79 stronger than serum 5 which is in agreement with the IgE titre data but against the Pru p 3 recognition pattern (Fig. 7.1). In the same

way, serum 2 showed two very faint bands corresponding to Pru p 3 and its digestion product while serum 1 did not show any recognition. This result is also against the Pru p 3 recognition pattern (Fig. 7.1). However, these faint identifications may not relate to IgE binding and may be due to the background effects.

In the case of digestibility of Pru p 3 loaded with linoleic acid, the IgE recognition was found to be much weaker as compared to Pru p 3 in an unliganded form (Fig. 7.3).

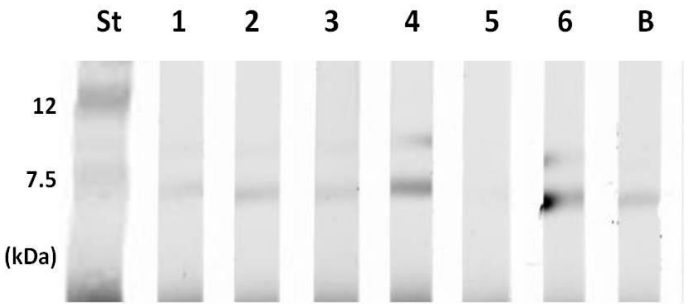


Fig. 7.3: IgE immunoblot of 120 minutes duodenal digestion of Pru p 3 loaded with linoleic acid. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

When Pru p 3 loaded with linoleic acid was digested, the IgE recognition decreased dramatically in case of serum 6, being the highest titre of IgE. Among all the sera, 4 and 6 weakly identified both Pru p 3 and its digestion product. To the rest of the blots, there are weak recognitions which was also found in blank showing a weak background recognition.

7.3.3 Effect of heat processing and simulated gastro-duodenal digestion of Pru p 3 on IgE reactivity

Heat treatment of Pru p 3 sharply decreased the IgE recognition as shown by immunoblot (Fig. 7.4). All the sera did not recognize heat treated Pru p 3 except for serum 6 which retained considerable IgE binding capacity. This is probably due to the fact that serum 6 had the highest IgE titre among the rest. Thus, on comparison between native and heat treated Pru p 3, heat treatment dramatically reduced IgE recognition.

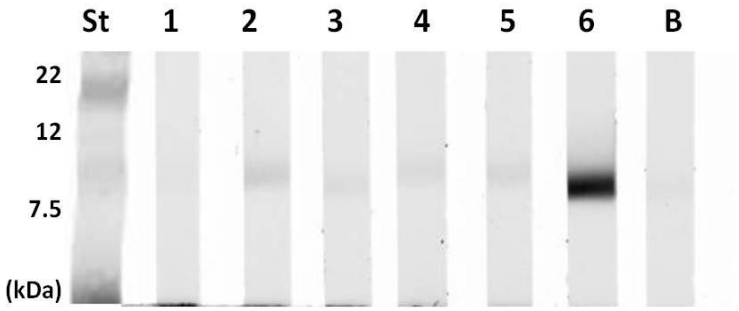


Fig. 7.4: IgE immunoblot of heat treated Pru p 3. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

In the case of heat treated Pru p 3 digested under simulated gastric and duodenal conditions, resulted in a complete loss of Pru p 3 IgE recognition at the end of both phases. None of the sera showed any recognition of digestion products of Pru p 3 neither the gastric nor the duodenal digests (Fig. 7.5).

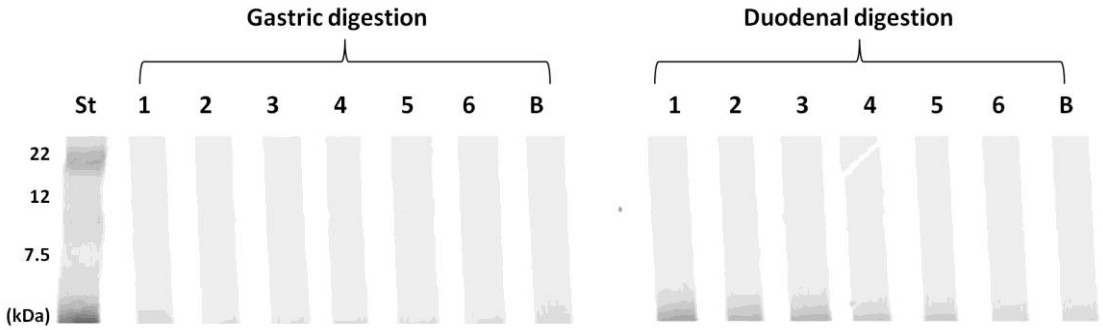


Fig. 7.5: IgE immunoblot of gastroduodenal digestion of heat treated Pru p 3. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

7.3.4 Effect of food matrix on simulated gastro-duodenal digestion and IgE binding of Pru p 3

Pru p 3 in peach peel was recognized by IgE from all sera after 2 minutes of oral digestion, identified as a single IgE reactive band in both the soluble fraction and the supernatant and the insoluble pellet fractions resulting from the model chew (Fig. 7.6). As might be expected given the lack of proteases in the simulated salivary fluid, no digestion product was seen in either of the phases.

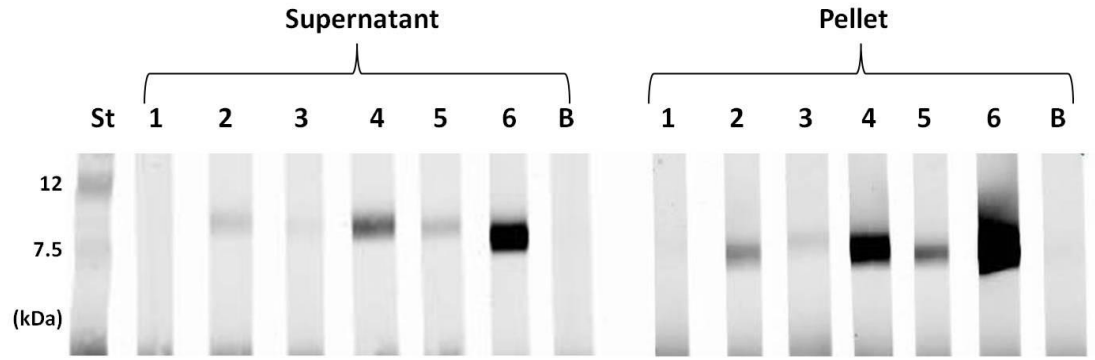


Fig. 7.6: IgE immunoblot of oral digestion of peach peel. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

In the oral digest of peach peel, higher content of Pru p 3 was found in the pellet as compared to supernatant, which was also confirmed by immunoblotting with rabbit IgG against Pru p 3 (Fig. 6.16). These results show that Pru p 3 is not completely released from the peel matrix during mild digestion conditions (2 U/mL salivary amylase, 2 minutes digestion). The greatest recognition of Pru p 3 was observed by IgE from serum 6 followed by serum 4 and 5 respectively. Between serum 2 and 3, serum 2 showed higher IgE recognition than serum 3. The results are similar to those expected from the IgE titre data and the binding pattern towards the purified Pru p 3 described above.

After 120 minutes of gastric digestion, Pru p 3 was found to be more extensively released into the supernatant from the pellet material as compared to oral digests, as shown by IgE immunoblotting (Fig. 7.7). However, the pellet material still had a higher content of Pru p 3 than the supernatant.

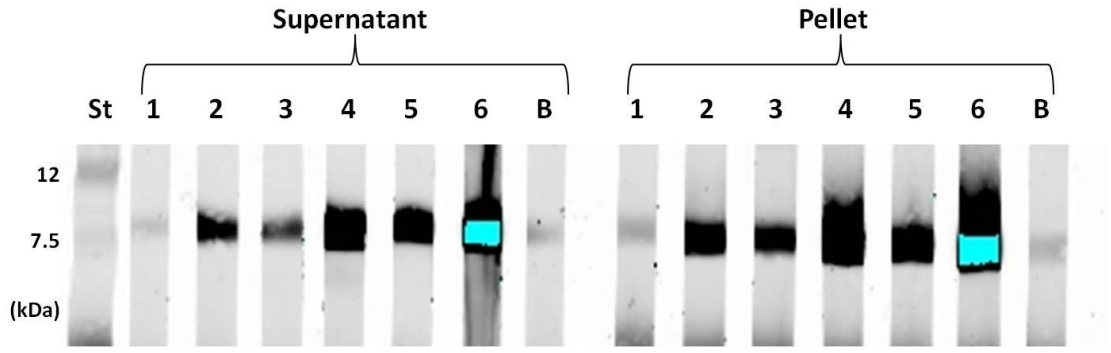


Fig. 7.7: IgE immunoblot of gastric digestion of peach peel. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

At the end of gastric digestion, IgE from all sera recognized Pru p 3 in both the supernatant and pellet material. Serum 6 again showed the strongest binding to Pru p 3 among the rest, followed by

serum 4 and 3 respectively. Between other two sera, serum 2 showed stronger recognition than serum 3, consistent with results presented above. The amount of Pru p 3 blotted from peel samples and recognized by the serum IgE was found to be higher in these samples resulting in a signal intensity beyond the detection limits of the gel imaging system. Therefore, the highest titre serum 6 showed a rectangular blue area in the centre of the band showing highest emission in this region. However, since the analysis undertaken was not quantitative, the qualitative recognition is discussed here.

After 120 minutes of duodenal digestion of peach peel, both Pru p 3 and its digestion product corresponding to residues 1-79 were identified by highest titre serum 6 (Fig. 7.8). Again, pellet material showed slightly higher amount of IgE reactive Pru p 3 as compared to supernatant.

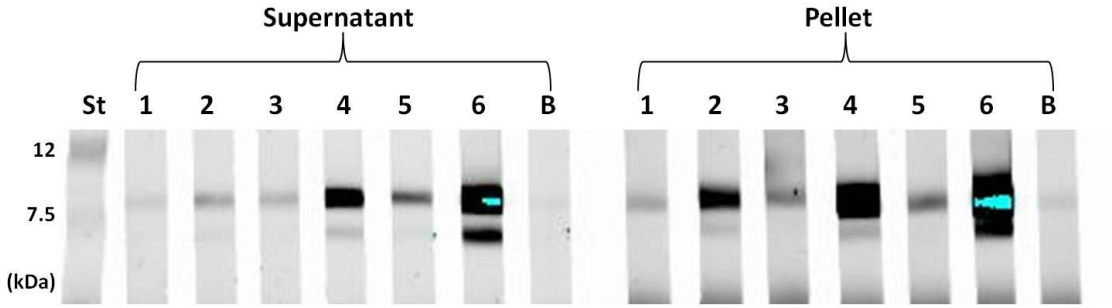


Fig. 7.8: IgE immunoblot of 120 minutes duodenal digestion of peach peel. St represents standard molecular weight markers. Lanes (1-6) represent sera (1-6) in Table 7.1. Serum 1 was from a non-atopic control. B represents a blot incubated with skimmed milk powder only.

On comparison between sera, IgE from serum 6 recognized Pru p 3 most strongly in the pellet rather than the supernatant. Both Pru p 3 and its digestion product were identified as IgE reactive bands in both the supernatant and pellet. Again, the amount of IgE binding to Pru p 3 exceeded the detection limit of gel imager showing some blue spots within the Pru p 3 bands. In the case of IgE from serum 4, it recognized Pru p 3 in pellet more strongly than in the supernatant while the digestion product was recognized only weakly. IgE from serum 5 recognized only Pru p 3 in both supernatant and pellet fractions but did not bind to the Pru p 3 digestion product. Similarly, IgE from serum 2 and 3 also recognized Pru p 3 only weakly but with stronger binding to Pru p 3 in the pellet than in the supernatant and without binding to its digestion products.

7.4 Discussion

The samples used in this study were the same those presented in chapter 5 and 6 of this study except native and heat treated Pru p 3. The 120 minutes of duodenal digestion samples of liganded and unliganded Pru p 3 were the same as mentioned in chapter 5. The gastric and duodenal digestion of heat treated Pru p 3 samples were also the same as mentioned in chapter 6. The oral digest, 120 minutes of gastric and duodenal digest samples were the same as mentioned in chapter 6. The patients' sera selected in this study represented a group of patients with different IgE titres to peach from group of patients with peach allergy from Spain and Italy selected for the FAST project which aims to develop immunotherapies for peach allergy based on Pru p 3. This was supplemented with sera from plasmaforesis samples and hence were not so well characterized.

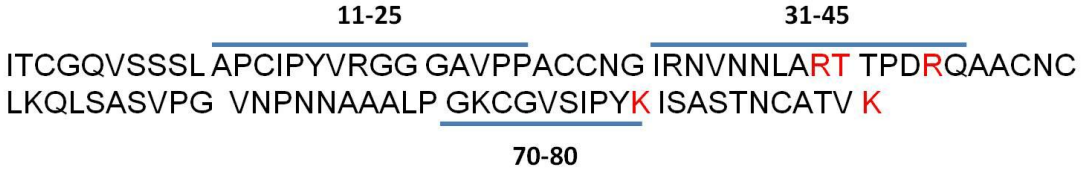


Fig. 7.9: IgE binding epitopes of Pru p 3. The continuous or sequential epitopes are represented as bar while discontinuous or conformational epitopes are represented in red colour. Adopted from (García-Casado et al., 2003).

All the sera showed IgE binding to purified Pru p 3 with varying degree which depended upon the titre to peach determined by immunoCAP. Unexpectedly, IgE from serum P0705 showed weaker recognition to Pru p 3 than P0725. This might be due to experimental error since IgE titre for P0705 was higher than P0725 or differences in the presentation of protein in immunoblotting experiments where the protein is reduced and denatured. There might also be a low efficiency of transfer of protein in this case which might be another reason for this low recognition. Since the protein is bound to nitrocellulose membrane, the recognition sites for this particular IgE might be physically less available to the sera and hence, low recognition was observed.

The simulated duodenal digestion products of Pru p 3 were also identified by all sera with varying intensities depending upon the IgE titre as given in Table 7.1. This is because of the fact that Pru p 3 initially digested into its large stable fragment 1-79 which contained nearly all the epitopes of Pru p 3 for IgE as given in Fig. 7.9. The most important conformational epitope, the triplet of residues 39/40/44 also lye in this region which remained intact in this large stable fragment. Further digestion products of Pru p 3 were residues 1-39, 40-79 and their further digestion products which splitted the epitopes resulting in no identification below Pru p 3 and 1-79 bands on blots (Fig. 7.2 and 7.3). The simulated duodenal digestion of Pru p 3 in the presence of linoleic acid as a ligand surprisingly showed weaker recognition of both intact Pru p 3 and its large stable fragment. This

may be due to either differences in the efficiency of transfer during immunoblotting with these samples or ligand binding to Pru p 3 but it does not alter the three dimensional structure to as great as extent where these results imply, and would not be retained following treatment with SDS and reducing agent (in preparation) (Abdullah et al., 2011). Similarly, the liganded protein may have effect on the mobility of the residues which could be the reason of this low recognition. This low recognition thus suggests that Pru p 3 might be less allergenic if transformed to its liganded form by using either food preparations or development of standard food processing steps to alter the behaviour of this protein.

The heat processing of the Pru p 3 strongly decreased the IgE recognition. This is due to the fact that heat processing destroyed the secondary structure of protein by unfolding and caused chemical changes to the protein (Fig. 6.1) and subsequent reduction of IgE recognition (Fig. 7.4). Since the conformational epitopes displaced there position as a result of heat denaturation, their recognition by IgE was reduced as seen here. Similarly, heat denaturation also buried the sequential epitopes which might also be bound to the nitrocellulose membrane and were less available to IgE for recognition. This also resulted in reduced IgE recognition by thermally denatured Pru p 3. Although this behaviour was seen on purified protein, it would be more useful to include the effect of food matrix on this study since we have shown in this thesis that Pru p 3 is more stable in peach peel than its purified form in solution. It might be expected here that heat treatment of peach peel still retains its allergenicity. This suggests that processing of peaches is very important in destroying the allergenicity of this highly consumed fruit. The digestibility of the heat treated Pru p 3 was improved in the simulated gastric and duodenal conditions for the same reason and hence further reduced the IgE recognition such that it was lost. However, this effect might not be seen during digestion of heat processed peaches in human since we have already seen that Pru p 3 is more stable when it is in its native food matrix i.e peach peel. Therefore, these results suggest that a further study is required to understand the allergenicity of various forms of peaches consumed either raw or processed.

The food matrix played an important role in affecting the resistance of Pru p 3 to simulated gastroduodenal digestion. This is demonstrated by the fact that Pru p 3 showed higher resistance to digestion in peach peel than when in a purified form (Fig. 6.16 and Fig. 5.7 respectively). It is difficult to compare the IgE recognition in the case of purified Pru p 3 digestion with that of peach peel digestion since the total amount of Pru p 3 blotted on the membrane is different and in the case of peach peel extract, not known. However, the result confirmed that major digestion product of Pru p 3 was recognized by IgE. For a comparative purpose, the peel extract should be quantified either by conventional means such as ELISA or a more sophisticated method such as MRM. Then equal

amount of Pru p 3 should be loaded to purified form which will provide clear conclusion about the effect of food matrix on IgE recognition.

The results thus need to include wider serum panel to confirm these findings and in need of further confirmations including studies such as histamine release assay to best understand the effect of food matrix and processing on the allergenicity of LTPs. The example of Pru p 3 has been discussed here, but it would be more useful to incorporate other examples of LTPs such as from apple and wheat.

8. General Discussion

The present study deals with determination of structural factors responsible for stability to processing, proteolysis and allergenic potential of LTPs. This study initially considered the structural stability of LTPs to processing and gastroduodenal digestion as the prime factor to determine its allergenicity. Ligand binding was considered to be responsible for increasing stability to proteolysis in GI tract. The food matrix was also considered to be responsible for such stabilities affecting the allergenic potential of these proteins.

Pru p 3 is the most frequent fruit allergen reported in Europe (Fernández-Rivas et al., 2003) while Tri a 14 is not very common (Inomata, 2009). Therefore, present study was designed to compare the structural features of these two model allergens to understand the structural behaviour of an LTP to be an allergen. The form of an LTP consumed may be very important in determining the possible allergenicity. For example, Pru p 3 is consumed mostly in raw form while Tri a 14 is consumed in most cases as a heat processed product. Similarly, the food matrix carrying this LTP may also be very important in determining the form in which these LTPs are delivered to human immune system either in the native or modified form. Similarly, the form of this delivery is also affected by the gut processing such as the proteolysis which affects the survival of IgE epitopes. The level of consumption is also very important in determining the tolerance to those LTPs such as Tri a 14 is consumed in a large amount as compared to Pru p 3 being not a part of staple food.

It has been reported that the structural stability of LTPs may be an important factor in determining the allergenicity of this class of proteins (Marion et al., 2007). The structural stability in this context refers to stability to harsh food processing conditions and to digestion in the GI tract. The findings presented in this thesis depart somewhat from these already accepted concepts. It was already known that many LTPs survive gastric proteolysis and thus are likely to pass into the duodenum in an intact form (Asero et al., 2000). In the case of stability to duodenal digestion, unfortunately no published data are available regarding the simulated duodenal digestion of at least one other LTP from *Rosaceae* fruit such as plum or apple to allow comparisons to be made with Pru p 3 here. However, this can be done for another fruit LTP, by taking the example of grape LTP (Vit v 1, (Vassilopoulou et al., 2006)). Comparisons with the gastroduodenal digestion of peach LTP (Pru p 3, (Cavatorta et al., 2010), this work), make it clear that Vit v 1 is more resistant to duodenal digestion, surviving for 120 minutes of digestion compared to Pru p 3, which disappeared after 60 minutes of duodenal digestion. In addition to the data published (Palacin et al., 2009) and presented in this thesis show that Tri a 14, like the barley LTP (Wijesinha-Bettoni et al., 2010) is also more stable to simulated gastroduodenal digestion than Pru p 3. This in turn demonstrates that both Tri a

14 and Vit v 1 should be more allergenic than Pru p 3, in contrast to the fact that Pru p 3 is reported to be the most allergic among these three LTPs. If susceptibility to digestion is considered to be related to allergenicity, our data does not support such conclusion and proposes that this susceptibility should not be the prime reason for determining allergenicity. While taking the example of Pru p 3 and Mal d 3, Pru p 3 is regarded as the most prevalent allergenic fruit among *Rosaceae* allergic patients which cross-reacts to Mal d 3 (Fernández-Rivas, 2009) contradictory to the fact that Mal d 3 is more stable than Pru p 3. These results suggest that Pru p 3 is more allergenic than Mal d 3 and Vit v 1 affecting larger population (Pastorello et al., 2003). Similarly Pru p 3 is more allergenic than Tri a 14 (Tordesillas et al., 2009). Therefore, stability to gastroduodenal digestion is not the sole key factor determining the allergenicity of LTPs. However, taking the example of a single LTP, it is obvious that intact protein and its digestion products, both are allergenic but the present study was a qualitative test and did not include the quantitative measures to see whether the IgE recognition of single intact protein band is equivalent to the combined recognition of the intact protein and its digestion product in case of the digested sample as seen here in case of Pru p 3.

Taking into account the IgE recognition of Pru p 3 digests by serum IgE from peach allergic patients, it is clear from the data that intact Pru p 3 and its large stable digestion product (1-79) are both recognized by IgE, suggesting that both intact and digested form of Pru p 3 retained its allergenicity. Therefore, stability to gastroduodenal digestion should not be considered to be the primary reason for an LTP to be allergic.

In this thesis, the effect of ligand binding has been comprehensively studied and the results suggest that stability to gastroduodenal digestion may be affected either by increasing or decreasing the proteolysis of LTPs. The effect however depends upon the fold and rigidity of the protein making it susceptible to proteolysis. We have shown in this study that ligand binding has improved the digestibility of Tri a 14 while did not affect Pru p 3. Our results also suggest that ligand binding may alter the IgE recognition of LTPs as demonstrated by the fact that liganded Pru p 3 with linoleic acid and its digestion product both showed reduced IgE reactivity. The allergenic potential of liganded LTPs thus need careful investigation before clear conclusions can be made. A careful molecular modelling of the protein structure supplemented with wider IgE binding studies, including immunoassays using a wider serum panel from LTP allergic patients are required to understand possible structural changes behind these interactions which may affect the IgE recognition of LTPs.

Protein characteristics are largely dependant upon their environment such as pH and modifying components such as sugars to glycate them. Consequently, the impact of the food matrix on the digestibility of LTPs was investigated. The results obtained suggest that Pru p 3 is more resistant to proteolysis in peach peel than in solution. This is due to the fact that oral digest taken in this study had physical interferences to enzyme availability as the system consisted most of the intact tissue which were not disrupted in simulated mastication. Therefore, a physical barrier (i.e. the plant cell walls) is hypothesized here being responsible for higher stability of LTPs to simulated gastroduodenal digestion. Other possible factors interfering the non-protein components of the matrix such as pectin, polyphenols, sugars, lipids, acids and fibres (Rawel et al., 2007) which may affect not only the native form of LTP but equally are available for digestive enzymes to modify them and affect their acitivities. However, the allergenic potential of the Pru p 3 itself was not affected as there was no impact of the matrix on recognition by IgE in both cases i.e in solution and in peel. Again, simulated gastroduodenal digestion also did not affect the IgE reactivity of intact and digestion product (1-79) of Pru p 3 being recognized both in solution and within peel by IgE. These results also suggest that effect of food matrix requires further investigations to confirm the effect on allergenic response and a clear conclusion is hard to suggest here. The effect of such matrices not only affect their bioavailability by creating physical interferences but may modify them resulting in loss of potential active sites to elicit immune responses.

In most cases, fruits are consumed fresh without any processing except for some processing such as peeling of banana or removal of seeds of mangoes and peaches. On the other hand, cereals are mostly consumed in their processed form such as wheat either in the form of baked bread or extruded pasta. The form of LTPs eaten are important in determining the allergenicity of these proteins. This is demonstrated by the fact that peach allergic patients can not tolerate even heat processed peaches such as canned fruit.

In case of structural stability of LTPs to harsh processing conditions such as thermal processing, it is possible to compare the properties of Pru p 3, Mal d 3 and Tri a 14, taken as examples. It has previously been reported that Mal d 3 is heat denatured at higher temperatures following prolonged heating, although it refolds upon cooling if heated to 90°C (Sancho et al., 2005) but does undergo irreversible denaturation at higher temperatures (Johnson et al., 2010) . In case of Pru p 3, previous reports (Gaier et al., 2008) and those presented in this thesis suggest that Pru p 3 is also heat denatured on heating above 90°C but this denaturation is irreversible. Pru p 3 does not refold upon cooling. This conclusion is also supported by our own finding by taking into account the structural stability of Pru p 3 and Tri a 14 to heat denaturation. In our own study, we proved that Tri a 14 is

more stable to heat denaturation than Pru p 3. Such stabilities demonstrate that Mal d 3 and Tri a 14 should be more allergenic than Pru p 3, contrary to the facts reported yet.

The allergenic potential of heat treated Pru p 3 has been studied here and was found that heat treated Pru p 3 showed reduced IgE recognition. It is clear from the results that heat denaturation of Pru p 3 is irreversible which shows a sharp decrease in IgE reactivity but not the full loss. These results suggest that heat treated Pru p 3 is still allergenic with reduced activity being consistent with the previous finding in case of Mal d 3 (Sancho et al., 2005). Such structural changes affected the epitope availability resulting in reduced IgE reactivity. Thermal denaturation of Pru p 3 displaced IgE reactive conformational triplet 39/40/44 from Pru p 3 surface resulting a dramatic reduction in IgE reactivity. Similarly, the sequential epitopes (Fig. 7.9) were also less available after heat denaturation either by overlapping with other residues or being bound to nitrocellulose membrane which also resulted in reduced IgE binding reactivity. These results further need a careful study to undertake molecular modelling studies to investigate the structural changes and the conserved regions after heat treatment which may be playing vital role in IgE reactivity. Therefore, resistance to thermal stability should not be regarded as the key factor to determine the allergenicity of a protein. This is because of the fact that although a protein is more allergenic when it is more stable to thermal denaturation, another protein wither higher stability might be less allergenic as discussed here with the example of Pru p 3 and Tri a 14. Although Tri a 14 is more stable than Pru p 3, it is less allergenic. Therefore, a more careful study is required to undertake to explore the possible reasons for a protein to be allergic and should not be relied on the data of a single protein characteristics rather should be incorporated comparative studies. However it is worth noting the stability to digestion is related to the rigidity of the protein structure, which may also be an important factor in determining the resistance of these proteins to thermal denaturation and in particular the importance of the disulphide bonds.

Given the effect of the food matrix on the susceptibility of native Pru p 3 to digestion, the effect of processing on the stability of LTPs in food matrix was also studied here by taking the example of Pru p 3 in peach peel. Results suggest that Pru p 3 is more resistant to thermal denaturation and gastric proteolysis in peach peel, being intact after 120 minutes of gastric digestion of heat treated peach peel. These results suggest either the protein is protected from thermal denaturation in the plant tissue matrix – for example proteins tend to have higher thermal transitions when heated at higher protein concentration. It may also be due to the matrix components mentioned above which may affect the digestibility and possibly the allergenic potential of LTPs by modulating their bioaccessibility and bioavailability. Thus these results suggest careful investigation of the effect of food matrices on the structural stability to proteolysis and allergenic potential of LTPs.

8.1 Future work

The present study not only dealt with the basic understandings of a food allergen and created several questions which require a broad investigation of allergens for them to be allergenic. In the first example, the stability to gastroduodenal digestion needs further investigations to understand the effect of such digestions on IgE epitope survival. These results clearly need a thorough molecular modelling study to conduct and find the answers supplemented with experimental procedures to prove the conclusions. The proposed plan is to determine the effect of the gastroduodenal conditions on protein fold and the charge states of various residues present at protein surface and its relation to the IgE reactivity. Secondly, the effect of proteolysis in simulated conditions on survival of known epitopes is also required to understand the stability and availability of intact epitopes. Finally, as the digested form of the protein remains intact by the intramolecular disulphide bonds, the flexibility of residues might be affected by this proteolysis and hence, a careful study is required to understand the possible interactions of these gut processed proteins with immune system.

The stability to thermal denaturation is also explained here which itself generated the questions regarding the direct relationship between such stabilities and allergenic potential. Again, a careful molecular modelling study is required to investigate such relationships supplemented with experimental data to confirm the conclusions made. The data presented here lacks the modelling studies showing the structural changes as a result of thermal processing and its effect on the localization of immunoactive IgE epitopes. Similarly, the effect of thermal processing on the modification of residues in epitope is also unclear which needs further investigations.

The effect of food matrix is also shown here with improvement in structural stability. These findings require the broadest investigations suggested in this chapter requiring the effect of each and every component of food matrix on the structural stability and its reaction to IgE reactivity taking the considerations of IgE epitopes known. For example, it is shown that pectin affects the digestibility (Polovic et al., 2007) but is not clear its effect on the epitope recognition. Similarly, the present data lacks the effect of glycation on LTPs on their immunoreactivity. Therefore, the effect of food matrix on allergenicity represents a very broad area which needs extensive research to understand a combined effect of food matrix on the delivery of LTPs to the immune system.

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